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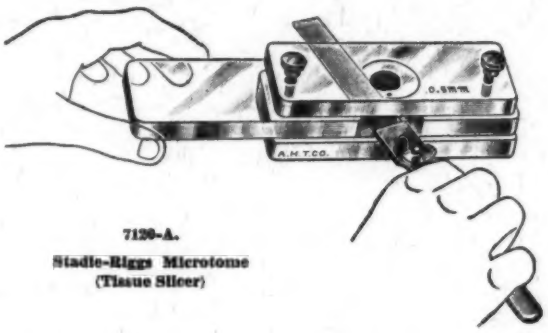

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THE SOURCES AND ACTIVITIES OF TWO CHROMATOPHOROTROPIC HORMONES IN CRABS OF THE GENUS SESARMA. II. HISTOLOGY OF INCRETORY ELEMENTS [1]

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In a previous paper dealing with physiological analyses concerning the activities and possible sources of chromatophorotropic hormones in three Japanese species of *Sesarma* (Enami, 1951), I have shown the sites of origin of two kinds of hormones, namely S and N hormones, on a gross anatomical scale. On the one hand, S hormone was concluded to be produced in both the sinus gland and all the principal ganglia, its occurrence in the latter being independent of the secretory activity of the former. This suggested a strong possibility of the universal existence in ganglionic tissues of a certain incretory mechanism essentially similar to that involved in the sinus gland.

On the other hand, N hormone was demonstrated to be much restricted in its distribution, only a few of the central nervous organs, *i.e.*, the brain and the medulla terminalis in the adults, the brain, the medulla terminalis, the commissural ganglion, and the thoracic ganglion in the juvenile forms, apparently producing the hormone. This suggested the presence in the said ganglia of specific incretory elements not as widely distributed as the presumed sources of the S hormone.

A histological study was carried out in order to determine the ultimate sources of the two hormones as well as the cytological details of their elaboration. The present work also includes a closer investigation of the sinus gland, whose secretory activity has hitherto been studied only superficially, in spite of the generally admitted significance of the gland as the predominant incretory organ in crustaceans.

Materials and Methods

Observations were mainly based on histological sections of tissues of the adults of *Sesarma dehaani*, weighing 25–30 g. and showing no sign of ecdysis (*cf.* Enami, 1951). For the purpose of the present study standard sections were prepared from the whole soft eyestalk tissues carefully freed from the exoskeleton, the isolated sinus gland, and the body ganglia such as the brain, the commissural ganglion, and the thoracic ganglion dissected out separately. The tissues were fixed in Heiden-

[1] This work was carried out at the Mitsui Institute of Marine Biology, Shimoda, Japan, with the support of a grant-in-aid from the Ministry of Education of Japan in 1948.

hain's Susa for 4 to 8 hours, and paraffin sections 8–10 μ thick were stained with Mallory's triple stain.

For specific purposes, especially for a detailed examination of the sinus gland, various other techniques including both vital and supravital staining were employed, references to which will be made in the following accounts.

Information obtained in the adults of *Sesarma dehaani* was compared with that in the adults of two other species of the same genus, *viz.*, *S. intermedia* and *S. haematocheir*, with corresponding results. Accordingly, the following account applies to all these species.

Observations

Histological signs of an incretory activity in the adults of *Sesarma* were observed in the following tissues and cells:

(1) Sinus gland
(2) Neurilemma of all ganglionic tissues examined
(3) Specific nerve-cells located within certain ganglia. Among these, three types can be distinguished as to their secretory behavior; they are here tentatively designated as α, β, and γ neurosecretory cells, respectively.

Thus, altogether five kinds of incretory elements are dealt with, their topography being diagrammatically illustrated in Figure 1.

1. The sinus gland and its secretory behavior

The sinus gland of *Sesarma* is well developed and, in fresh or even in formalin-fixed eyestalks, is macroscopically visible owing to its well-known characteristic opacity and slightly bluish hue (Brown and Cunningham, 1939, etc.). It is located at the dorsal aspect of the junction between the medulla interna and the medulla terminalis (Fig. 1, SG). Its outline roughly conforms to a triangle, one of whose apices is tapering off in a proximal direction. When viewed through transmitted light, the gland appears brownish yellow in marked contrast to the almost colorless nervous tissues nearby. The approximate volume of the gland was measured in animals of different sizes by means of a simple calculation from the area of optical section and the thickness of the isolated gland sandwiched between the slide and cover glass of a Thoma-Zeiss hemacytometer (Fig. 2).

The proximal tapering indicates the innervation by the sinus-gland nerve which is traceable along the frontal surface of the neuropile of the medulla terminalis up to a disc-like cluster of giant nerve-cells located at the proximal ventral-lateral corner of the medulla (Fig. 1, A). The nerve leaves the nerve-cell group at the center of the latter's internal concavity, and within a short distance from the point of its departure sends off a slender branch proximally, which enters into the pedunculus lobi optici (Fig. 1). Concerning the innervation of the gland in *Cambarus*, Welsh (1941) has described, besides the unquestionable innervation by the sinus-gland nerve originating in the medulla terminalis, a distribution to the surface of the gland of a branch of the oculomotor nerve II, a fact which demonstrates a dual innervation of the gland. However, as far as the present study is concerned, the innervation of the sinus gland of *Sesarma* takes place by the sinus-gland nerve exclusively, no other routes having been found, although some of the finer branches

of the oculomotor nerve II, in supravital staining with methylene blue, appear to be in superficial contact with the glandular tissue.

The glandular tissue is, just as described in the sinus glands of various other crustaceans (Hanström, 1933, 1937; Sjögren, 1934; Ståhl, 1938; Carstam, 1941; Pyle, 1943; etc.), composed of a folded continuous lamella of syncytium, including a variable amount of large and small acidophile droplets. Several branches of the dorsal radial blood-sinus are enclosed, a continuous structureless membrane of about 0.3 μ thickness separating the glandular lamella from the blood-sinus (Fig. 3, L.M.). This limiting membrane was made visible in deep blue color by means of Mallory's triple staining after fixation with 5% sulfosalicylic acid. It is noteworthy that,

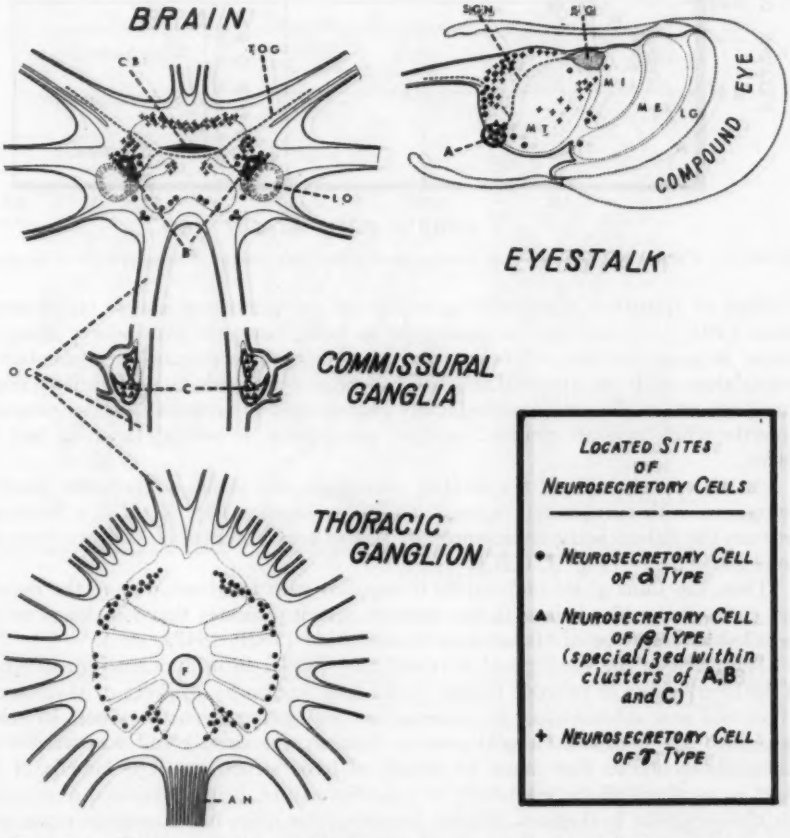

FIGURE 1. Diagram of the central nervous organs and the eyestalk tissues of *Sesarma* showing distribution of incretory elements. AN, abdominal nerve; CB, central body of the brain; F, foramen; LG, lamina ganglionaris; LO, lobus olfactorius; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OC, oesophageal commissure; SG, sinus gland; SGN, sinus-gland nerve; TOG, tractus olfactorio-globularis.

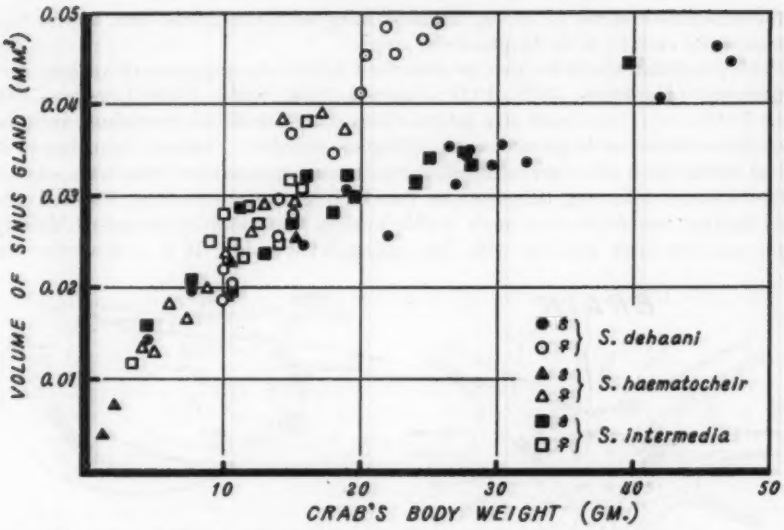

FIGURE 2. Volume of the sinus gland plotted against the body weight in three species of *Sesarma*.

contrary to Hanström's statement pointing out the perforated nature of the membrane (1937), I observed the membrane as being complete everywhere along its course in good sections. I repeatedly examined the membrane in a number of preparations with the result that what had been dealt with as perforations might have been one of the artefacts frequently encountered in sections as those prepared from the whole eyestalk enclosed by hard exoskeleton, to which Hanström had resorted.

At the opposite side of the limiting membrane, the tissue of the sinus gland is contiguous with surrounding spongy connective tissue (Fig. 3, CT), a landmark between the tissues being represented by sets of coarse networks of fibers from the sinus-gland nerve (Fig. 3, E.B.).

Thus, the sinus gland of *Sesarma* is supplied with innervation from the outside and encloses the blood-sinus in the interior, which indicates that it belongs to the so-called inverse type of Hanström's classification (1937, 1947a, etc.).

The tissue of the sinus gland is remarkably similar in its fundamental structure to the neurilemma of nervous tissues, a fact long known by workers of Hanström's school and now substantiated by observations with preparations in which, by treatment with 5% hydrochloric acid prior to fixation, the sinus gland was freed from acidophile inclusions that mask the details of basic structure. The lamella of the gland is, as stated above, essentially of syncytial nature, being composed of continuous chromophobe hyaloplasm divided into irregular units by incomplete septa consisting of coalesced and half-coalesced granules (Fig. 3, S). Within each of the units are a variable number of nuclei of different appearance. Such characteristics are in complete accord with those observed in the neurilemma tissue (Fig. 4). Surrounding the blood-sinus, units of the sinus-gland syncytium are cylindrical, standing vertically against the limiting membrane (Fig. 3).

A remarkable difference between the sinus gland and the neurilemma exists in regard to the amount and morphological variability of the acidophile inclusions, the sinus gland predominating in these respects. As to configuration, stainability, plasticity, microscopic composition, etc., three types of inclusions within the sinus gland are distinguishable which are here tentatively dealt with as colloids A, B, and C, respectively.

Colloid A (Fig. 3, middle figure, CA). Ovoid or ellipsoid masses ranging from 4 μ to 8 μ in length, stained most intensely with the acid fuchsin component of Mallory's triple stain. It is found either in isolation or in small aggregations, its

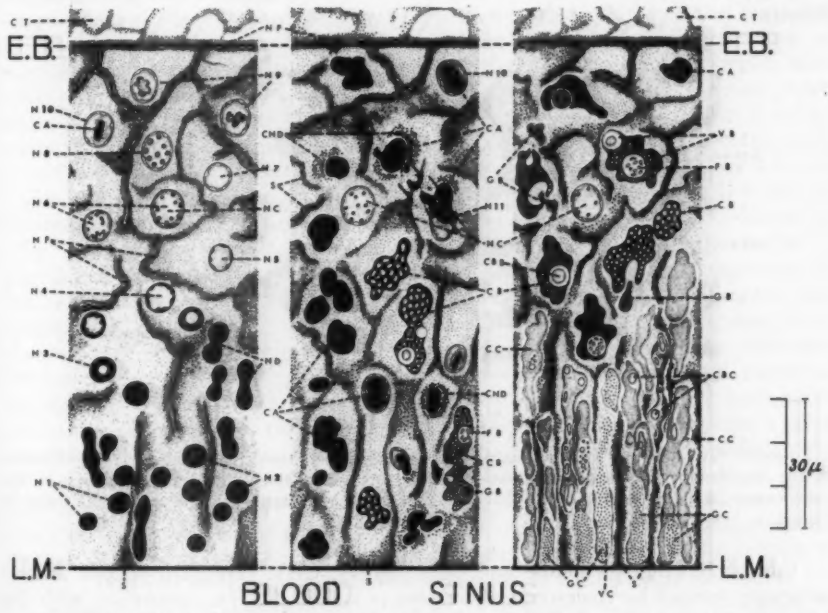

FIGURE 3. Histology of the sinus gland illustrated semi-diagrammatically on the basis of studies with sections and supravital mounts. The whole process of secretory behavior is, for the sake of convenience, arranged in three separate views, of which the left one represents the process of transformation of the nucleus, and both the middle and the right ones are indicating the process of transformation of secretory precursor derived from the nuclear change. E.B., external boundary of the glandular tissue; L.M., limiting membrane.

Left figure: N 1–N 10, successive states of nuclear change; NC, nucleus most commonly seen in the gland; ND, nucleus showing direct division; CA, colloid A formed within the nucleus; NF, nerve-fibers; CT, connective tissue; S, incomplete septum of syncytium.

Middle figure: N 11, liberation of colloid A from nuclear capsule; CND, nuclear capsule in the process of disintegration; CB, colloid B; FB, fragmentary inclusion in the vacuole of colloid B; GB, intra-vacuolar granules of colloid B; other notations are the same as those in the left figure.

Right figure: VB, vacuole in colloid B; CBB, central body in the vacuole of colloid B; CC, colloid C; CBC, central body in the vacuole of colloid C; GC, granules in the vacuole of colloid C; VC, vacuole in colloid C; other notations are the same as those in the left figure.

occurrence being inconsistent at different spots of the same glandular lamella. In the supravital state the colloid shows a marked affinity to alizarine S, the resulting coloration being associated with pronounced refractility, and it displays considerable plasticity, which suggests a high viscosity of its composite material. In both fixed and *in vitro* preparations, its staining is homogeneous, no structural differentiation being present. Characteristic staining reactions of the inclusions are further observed in sections impregnated with silver according to the techniques of Bielschowsky and of Hortega, in which the colloid reduces silver almost selectively. In sections treated with $N/4$ sodium hydroxide and stained with Janus green B, the colloid appears deep greenish blue in contrast to the unstained surrounding tissue elements.

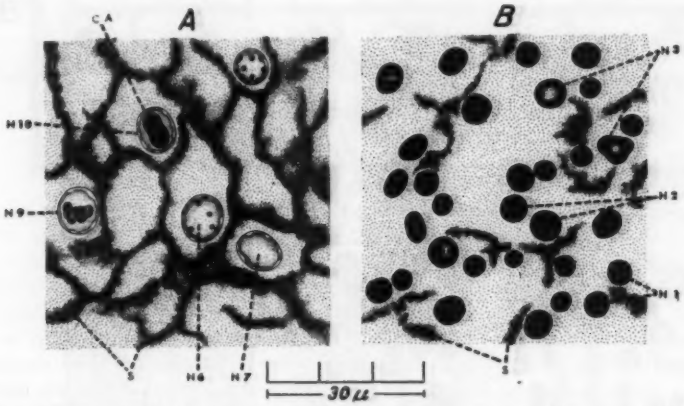

FIGURE 4. Histology of the neurilemma semi-diagrammatically illustrated. A—Portion of the neurilemma showing formation of colloid A within the nucleus. B—Portion of the neurilemma including a crowd of small basophile nuclei. Notations are the same as those in Figure 3.

Colloid B (Fig. 3, middle and right figures, CB). Irregular-shaped masses seemingly formed by coalescence of masses of colloid A. In comparison with the latter, it shows weak stainability with both acid fuchsin in Susa-Mallory preparations and alizarine S applied *in vitro*. Together with the lowering of the affinity to these dyes, a marked decrease in the power of silver reduction together with the stainability with Janus green B is characteristically observed with this kind of glandular inclusion. In contrast to the apparent homogeneity of colloid A, colloid B is differentiated into a feebly acidophile matrix substance which appears less viscous than colloid A, and almost chromophobe contents of various sizes and amounts. Vacuoles occur in the colloid (Fig. 3, right figure, VB), which remain achromatic in Susa-Mallory preparations but are selectively stained with trypan blue, anilin blue, Congo red, eosin and tropeolin *in vitro*. They may or may not include formed elements, whose appearance ranges from single large chromophobe bodies (Fig. 3, right figure, CBB) to several irregular achromatic fragments (Fig. 3, middle and right figures, FB) and a number of granules deeply stained with anilin blue in Susa-Mallory sections (Fig. 3, middle and right figures, GB). Such vacuolar

inclusions are unstainable *in vitro,* but are clearly demonstrated when their surroundings are stained with the dyes mentioned above. Irrespective of their size, no sign of Brownian movement is observed *in vitro,* which is suggestive of considerable viscosity of their surroundings within the vacuoles. It was not infrequent to observe in both sections and supravital mounts of the sinus gland that the said anilin-blue granules together with their surrounding viscous substance were on the way of freeing themselves from colloid B into the hyaloplasm of the glandular tissue (Fig. 3, right figure).

Colloid C (Fig. 3, right figure, CC). Almost formless masses which, by coalescence, grow to larger forms conforming to the contours of the divisions of the glandular tissue. In Susa-Mallory preparations, this kind of inclusion is stained fairly well and homogeneously with Orange G, no morphological differentiation of composition being obvious. In whole mounts of the sinus gland *in vitro,* a wide affinity of the colloid to basic dyes such as Dahlia, Cresylecht violet, Gentian violet, methylene blue, and neutral red was disclosed, and an evident sign of its low viscosity was its tendency to assume a rounded appearance. In contrast to both colloid A and colloid B, this colloid rejects alizarine S *in vitro,* and in sections it reacts negatively to silver impregnation and also to staining with Janus green B following treatment with NaOH. Moreover, it is characterized by active vacuole formation (Fig. 3, right figure, VC). In vacuoles of various sizes one finds a number of granules of uniform size (Fig. 3, right figure, GC), several irregular fragments, or one or several central bodies (Fig. 3, right figure, CBC), all of which remain unstainable in both fixed and supravital conditions. *In vitro,* such vacuolar inclusions exhibit more or less pronounced Brownian movement, the smaller their size, the more active the visible motion. This fact indicates a certain fluid nature of the vacuolar content surrounding the inclusions. The development of such vacuoles in this colloid is very pronounced, and there are figures indicating that a given mass of the colloid may be almost wholly occupied by an enlarged vacuole.

Possible transformation of secretory materials within the sinus gland. The features described above are characteristic of the three representative forms of colloid inclusions of the sinus gland, but there are other forms derived from the respective colloids, which lie either between colloids A and B or between colloids B and C. This fact indicates a possible continuity of all the colloidal inclusions, *i.e.,* the possibility of a transformation of colloid A to colloid C by way of colloid B. In relation to such a possibility, the following observation appears to be of interest.

Upon treatment with 10% pyridine, a freshly isolated sinus gland loses most of its characteristic opaque whiteness in less than 30 minutes. Formalin-fixed and Mallory-stained sections of such material reveal that the vanishing of whiteness is due to the almost complete disappearance of colloid C. Nevertheless, both colloid A and colloid B are known to be resistant to the treatment. Of these remaining inclusions, colloid A as a whole retains a remarkable affinity to acid fuchsin, while colloid B reacts to the dye in a manner different from that shown by the one not treated with pyridine. The fragmentary inclusions within colloid B take the dye almost selectively, whereas the homogeneous ground substance around them is but feebly stained. That is, the affinity to acid fuchsin is reversed in the two kinds of components of colloid B as a result of the treatment with dilute pyridine.

After all, there are indications that the initially highly viscous and homogeneous

mass (colloid A) differentiates into a less viscous substance as the result of progressive disintegration into finer particles, which further changes into a more fluid substance as the result of a separation of viscous substance by means of vacuole formation. The fluid substance thus produced (colloid C) may then change into intravacuolar fluid and granules, both of which appear to be the ultimate products of transformation of colloid A, taking place within the tissue of the sinus gland.

Derivation of secretory material from nucleus. Inasmuch as the acidophile inclusions of the sinus gland are considered as secretory material (*cf.* Hanström, 1937, etc.), the preceding account of the possible fate of colloid A throws some light on the process of formation of the active material. Another problem, so far left untouched, concerns the origin of the glandular inclusions.

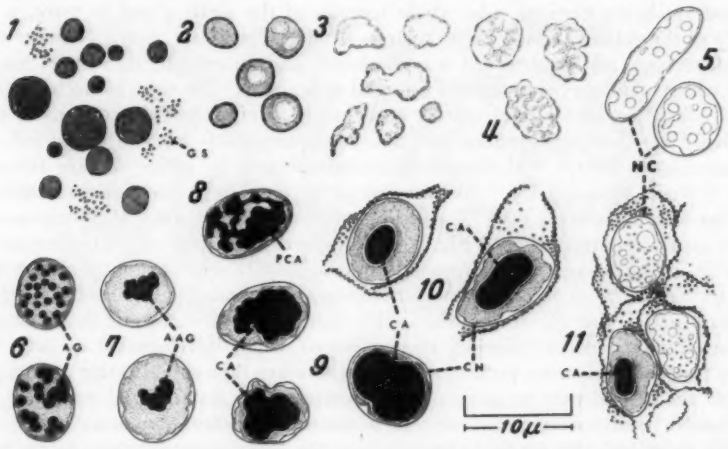

FIGURE 5. Variety of nuclear figures in both the sinus gland and the neurilemma. Susa, Mallory. NC, common appearance of nucleus; GS, granules of septum of syncytium; AG, globules stained with Orange G; AAG, aggregated Orange G-globules; PCA, precursor of colloid A; CA, colloid A; CN, capsule of nucleus surrounding colloid A.

It was not infrequent to observe in both sections and supravital mounts of the sinus gland that colloid A in its isolated form was enclosed by an ovoid or ellipsoid capsule of considerable thickness which, although being refractory to any dyes, is visible on account of its high refractility (Fig. 3, left and middle figures, N 10; Fig. 5, *10*). Some figures showed the release of the colloid masses from their respective capsules through rupture of the latter (Fig. 3, middle figure, N 11; Fig. 7, *2–5*). In areas of the glandular lamella where such figures were found in close aggregation, freed masses of the colloid were seen in coalescence with each other to form larger masses which were associated with empty capsules (Fig. 7, *1*). The capsule, after releasing the colloid mass, appeared to disintegrate, being at first decomposed into irregular pieces and eventually reduced to finer granules (Fig. 3, middle figure, CND; Fig. 7, *6–9*).

In the state of full integrity, the encapsulated body conforms in size and form

to the nucleus, a fact which led me to an inquiry regarding the role of the nucleus of the sinus gland. In the routine hematoxylin-eosin preparations, the nuclei of the gland most commonly met with are of vesicular appearance, containing varying amounts of basophile granules which may be taken for granular chromatin, but no acidophile elements corresponding to nucleoli. In sections treated after Feulgen's method modified by Rafalko (1946),[2] it was found that the chromatin or, more precisely, the basophile elements are arranged at the periphery of the nucleus, the latter's interior being occupied by chromophobe caryoplasm (Fig. 3, NC or N 6; Fig. 6, *7*). Besides this appearance there were other features of the nucleus, indicating its derivation from a smaller structureless basophile body.

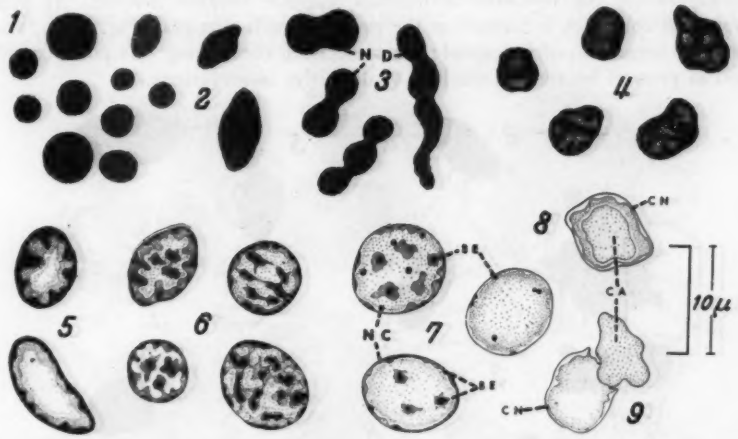

FIGURE 6. Figures of nuclei in both the sinus gland and the neurilemma. Helly, Feulgen (Rafalko's modification). ND, amitotic figures of nuclear division; NC, common appearances of nuclei. BE, basophile elements at the periphery of nucleus; CA, colloid A; CN, capsule of nucleus.

What is designated to be the original form of the nucleus is a minute sphere, ovoid or ellipsoid, of dimensions ranging from 3 μ to 6 μ, which stains with hematoxylin, safranin and muci-carmine and also exhibits a marked uniform reaction with Feulgen's stain except for a thin peripheral area (Fig. 3, left figure, N 1; Fig. 6, *1* and *2*). In Susa-Mallory preparations, such a body takes anilin blue uniformly, but the peripheral area predominates in this stainability (Fig. 5, *1*). Nuclei of such structureless appearance were at times found in dense aggregation in certain restricted areas of the sinus gland, where they were observed in association with groups of melanin granules, and occasionally in the process of direct division (Fig. 3, left figure, ND; Fig. 6, *3*).

Such a state of the nucleus appears to change to a state in which the chromophobe portion is differentiated in the center of the basophile mass (Fig. 3, left figure, N 2 and N 3; Fig. 5, *2*; Fig. 6, *4*), and there were figures showing further successive

[2] Use of this technique was aided by the kind information received from Dr. K. W. Cleland, Sydney University, Australia.

changes, in which the nucleus becomes enlarged by the expansion of the central chromophobe mass which is likely to take place at the expense of the basophile portion restricted to the periphery (Fig. 3, left figure, N 4 and N 5; Fig. 5, *3* and *4*; Fig. 6, *5* and *6*). These changes seem to result in the common vesicular state of the nucleus already referred to.

Figures were observed indicating the formation of strongly acidophile droplets within the chromophobe substance of the nucleus, which stained with the Orange G component of Mallory's stain and in this respect resembled the ordinary nucleoli (Fig. 3, left figure, N 8; Fig. 5, *6*). In some of the nuclei, such droplets showed a marked tendency to coalesce (Fig. 3, left figure, N 9; Fig. 5, *7*), and along with this phenomenon the refractile achromatic capsule became evident. It appears probable that colloid A is formed as the result of such changes (Fig. 5, *8*), accompanying the formation of its capsule, but a decision concerning this point cannot be reached at present because of the lack of definitive observations.

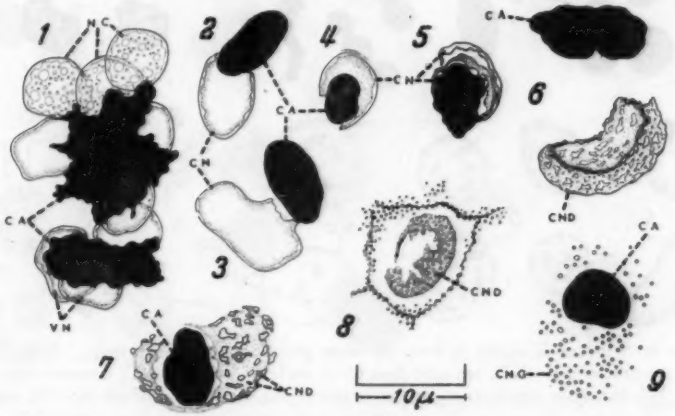

FIGURE 7. Figures showing liberation of colloid A from the nucleus and the later fate of nuclear capsule in the sinus gland and the neurilemma. Formalin, *N*/4 NaOH, Janus green B. NC, common forms of nuclei; CA, colloid A; VN, vacant nuclei; CN, capsule of nucleus; CND, disintegrating capsule of nucleus; CNG, granules resulted from disintegration of nuclear capsule.

2. Secretory activity of the neurilemma

Just as in the sinus gland, the nucleus plays a significant role in the production of acidophile inclusions in the neurilemma. The neurilemma of all ganglionic tissues was observed to produce a kind of colloid which was morphologically quite similar to colloid A of the sinus gland (Fig. 4, *A*, CA). In Susa-Mallory preparations or more clearly in sulfosalicylic acid-Mallory preparations of each of the principal ganglia, the colloid in question was found in varying amounts in different portions of the neurilemma, whose brilliant red coloration stood out impressively from the light blue tinge of the background. It was further observed that what was known about the morphological change of the nucleus within the sinus gland in relation to the process of the formation of colloid A was also the case regarding the nucleus of the neurilemma (Fig. 4). Such facts indicate that the same secretory mechanism is

operating in the two kinds of tissue in question, which, as stated above, are remarkably similar in their fundamental structure.

However, the neurilemma was observed to be inferior to the sinus gland with respect to the nuclear activity, and, moreover, present investigation failed to ascertain the transformation of colloid A into other forms, because of the apparent absence of both colloid B and colloid C or their homologues within the neurilemma tissue.

3. The neurosecretory cells

The present study includes the search for possible incretory elements in the nervous tissues outside the neurilemma, which, as already stated, resulted in the finding of three kinds of neurosecretory cells.

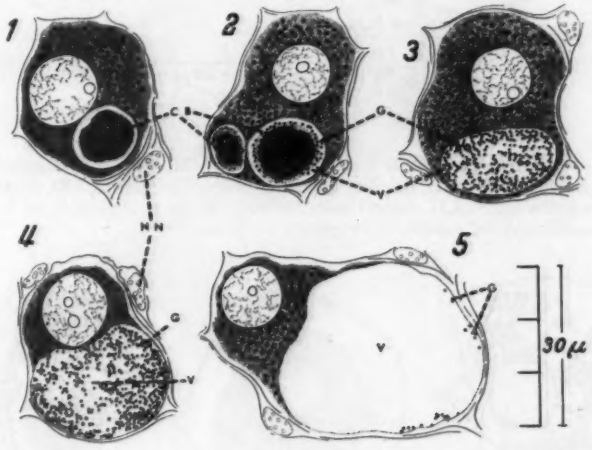

FIGURE 8. α neurosecretory cells from the brain and the medulla terminalis. Susa, Mallory. CB, central body formed in vacuole; G, granules derived from the central body; V, vacuole; NN, nucleus of the neurilemma.

α *neurosecretory cell* (Fig. 8). Secretory behavior was observed in some unipolar nerve-cells of the common type widely distributed in the brain, the medulla terminalis, the medulla interna and the medulla externa as well as the thoracic ganglion (Fig. 1). Excepting those found in the thoracic ganglion which were represented by giant cells attaining about 60 μ in length and including a large vesicular nucleus of about 20 μ diameter with one or two nucleoli of about 4 μ, the cells in other ganglionic tissues are generally of moderate size, measuring about 30 μ in length and including a nucleus of about 12 μ in diameter in which one or two nucleoli of about 2 μ are present. Irrespective of the difference in cellular dimensions, all of the cells are rich in cytoplasm, in which significant vacuole formation takes place. In Susa-Mallory preparations of the said ganglia, formation of a large fuchsinophile body within each vacuole (Fig. 8, *1*), splitting of the body into a number of fuchsinophile granules (Fig. 8, *2*), and dispersion of isolated granules within the

largely expanded vacuole (Fig. 8, *3* and *4*) were seen. Sometimes, expanded vacuoles were observed to be almost free of formed inclusions except for a few fuchsinophile granules (Fig. 8, *5*). With the formation of such vacuoles, the cytoplasm of the cells exhibited a variable affinity to acid fuchsin; the cytoplasm rejected the dye in the presence of many fuchsinophile granules within the vacuole, but was

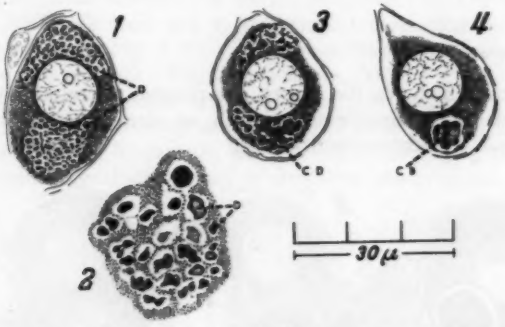

FIGURE 9. β neurosecretory cells from the brain and the commissural ganglion (*cf.* Fig. 1, B and C). Susa, Mallory. D, droplets appearing within cytoplasm; CD, droplets coalesced to form homogeneous mass; CB, central body formed. Scale is applicable to 1, 3, and 4. 2 is a further magnification of portion of cytoplasm in 1.

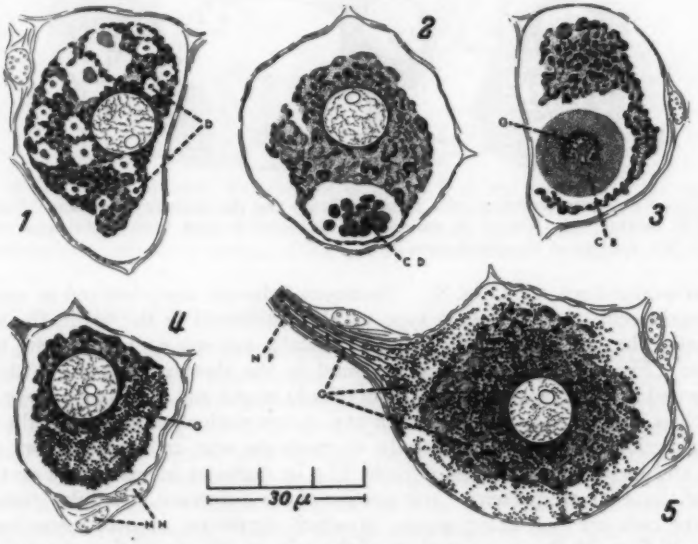

FIGURE 10. Giant β neurosecretory cells from the medulla terminalis (*cf.* Fig. 1, A). Susa, Mallory. NF, nerve-fiber innervating the sinus gland; NN, nuclei of the neurilemma; G, secretory granules; other notations are the same as those in Figure 9.

stainable in higher or lower degrees when the vacuole was deprived of most of its granular inclusions. Pictures were observed indicating a possible absorption of the intra-vacuolar granules by the cytoplasm, a fact which might explain the fuchsinophilic character of the cytoplasm during the disappearance of the granules.

β neurosecretory cell (Figs. 9, 10, and 11). The second type of neurosecretory cells differs from the preceding one in its mode of secretory activity and in the predominant affinity of the secretory material to the anilin blue component of Mallory's stain and also in the mode of secretory behavior. Cells showing this type of secretion were detected in the brain, the medulla terminalis and the commissural

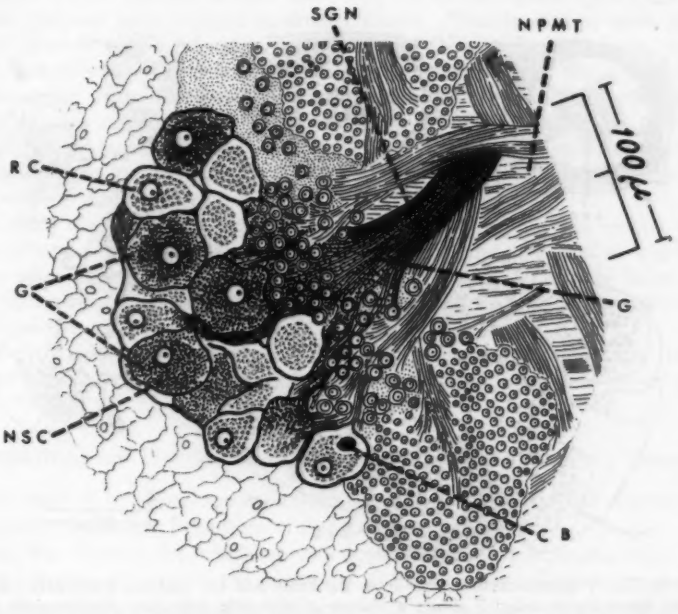

FIGURE 11. Section of the cluster of giant β neurosecretory cells of the medulla terminalis (*cf.* Fig. 1, A). Susa, Mallory, sagittal to axis of the eyestalk. SGN, sinus-gland nerve; NPMT, neuropile of the medulla terminalis; G, secretory granules; RC, resting cell; NSC, cell in secretory activity; CB, central body formed in the cell.

ganglion, but not in other ganglia. In the brain, such cells were located exclusively in a group of nerve-cells situated just anterior to the lobus olfactorius (Fig. 1, B). They, together with those found in the commissural ganglion (Fig. 1, C), are of moderate size, measuring ca. 30 μ in length and including a vesicular nucleus of ca. 10 μ in diameter (Fig. 9). Their cytoplasm is fairly homogeneous and of compact appearance, showing but slight contraction upon fixation. The cells belonging to the medulla terminalis are represented by a number of giant unipolar cells which constitute the conspicuous cluster at the ventral surface of the proximal portion of the neuropile of the medulla, dispatching nerve-fibers innervating the sinus gland

(Fig. 1, A; Fig. 11). These cells are composed of loose cytoplasm which, upon fixation with Bouin, Allen-Bouin, formalin, Helly, sulfosalicylic acid, Susa, and Zenker, contracts more or less conspicuously. The average dimension of the cells, measured by the contour of the surrounding neurilemma capsule, was about 50 μ, and the nucleus attained about 15 μ in diameter (Fig. 10).

In the cytoplasm of the β-type neurosecretory cells occurred many droplets feebly stained with anilin blue (Fig. 9, *1* and *2*; Fig. 10, *1*). Also observed in the cytoplasm of some cells were one or two spherical bodies taking up anilin blue considerably, which seemed to result from coalescence of the said droplets (Fig. 9, *4*; Fig. 10, *3*; Fig. 11, CB). Each of these spherules was observed to split into fine

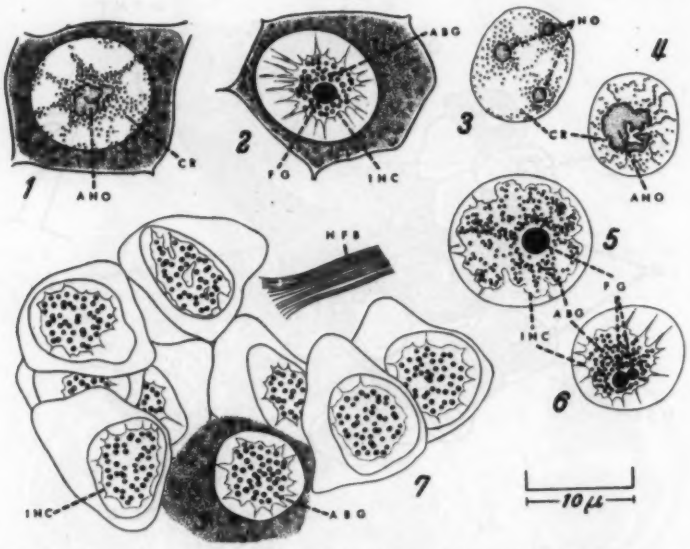

FIGURE 12. γ neurosecretory cells from the brain and the medulla terminalis. Susa, Mallory. 3–6 show nuclei only; 7 shows a cluster of the cells indicating synchronism of nuclear activity. ANO, nucleoli aggregated at the center of nucleus; CR, chromatin granules; FG, fuchsinophile globule; ABG, anilin-blue granules; INC, intra-nuclear colloid; NO, nucleoli; NFB, nerve-fiber bundle.

granules stained deeply with the dye (Fig. 10, *3* and *4*). The resulting granules were dispersed over the whole area of the cytoplasm, and it was of much interest to find in a few instances that they were present also along the path of axons arising from the cells with secretory activity (Fig. 10, *5*; Fig. 11, G). In one case, the granules were detected along the course of the sinus-gland nerve for approximately two-thirds of its whole length.

γ neurosecretory cell (Fig. 12). Unlike the preceding two types of neurosecretory cells, the third type is characterized by a kind of nuclear secretion. In some small unipolar nerve-cells measuring about 20 μ in length, as well as in some of the cells belonging to the so-called globulus type, measuring about 10 μ in length,

the nucleus underwent a series of changes unassociated with any visible change in the cytoplasm. There were pictures showing formation of a large intra-nuclear droplet uniformly stained with the Orange G component of Mallory's stain, within some of which were differentiated a fuchsinophile spherule and many anilin blue granules (Fig. 12, *2* and *5*). The fuchsinophile spherule was different from the nucleolus in the ordinary state of the cells in question in that it was far larger than the latter and was stained conspicuously with acid fuchsin in contrast to the latter's affinity to Orange G. Similarly the anilin blue granules were different from the granular chromatin in size and in stainability, the former far exceeding the latter in this respect. Other figures showed that both the fuchsinophile spherule and the anilin blue granules were reduced to smaller forms. That is, two or more globules developed from a single spherule, which, unlike their preceding form, took up Orange G considerably and resembled nucleoli, and a number of fine granules were derived from the anilin blue granules, which resembled the chromatin (Fig. 12, *6*). These figures are suggestive of a reduction of intra-nuclear inclusions to nucleoli and chromatin granules. As regards the process of formation of the intra-nuclear colloid, there were indications suggesting its origin from a central aggregation of both the nucleoli and the chromatin (Fig. 12, *1*, *3*, and *4*).

Such being the case, it would appear plausible that the nucleus of this type of nerve-cell shows a kind of reversible change, and that the secretion of a diffusible substance takes place in the course of the change. The present study failed to obtain any sign of discharge of formed elements from the nucleus to the surrounding cytoplasm of the cell.

Nerve-cells showing such nuclear change were located in abundance in the brain as well as in the medulla terminalis, but were scarcely detected in other ganglia (Fig. 1).

Considerations on Possible Sources of Chromatophorotropic Hormones

On the basis of the histological findings so far described, possible sources of the two chromatophorotropic hormones of *Sesarma* may be analyzed.

Taking into account the results of the experimental study (Enami, 1951) which have demonstrated that one of the hormones, S hormone, is originating not only in the sinus gland but in all the principal ganglia in the adults, one may attach some significance to the remarkable identity of the fundamental structure as well as the mode of secretion of the sinus gland and the neurilemma universally associating with the nervous tissues. These structures are equivalent in that their secretions appear to originate as the result of a transformation of the nucleus, although the fate of the secretory material within the respective tissues appears to be different. The secretory precursor, or colloid A, in the sinus gland undergoes a sequence of complicated changes to be transformed into a semi-fluid form (colloid C) which is further transformed through vacuole formation into liquid and granules. Now, in connection with the problem of what form the final secretion may assume, the observed completeness of the limiting membrane is to be taken into consideration. It appears possible that the final secretion containing S hormone is of diffusible nature, to which the limiting membrane is permeable, and that no kind of formed element can take its way out through the membrane, in which no sign of microscopic

perforation was observable. In this respect, the vacuolar fluid of colloid C rather than the co-existing granules appears to be of significance.

Concerning the role of the vacuolar contents of colloid B, which are liberated as a whole from the latter, no concrete information could be obtained. They may somehow be responsible for the formation of certain secretory materials, or they may be nothing but by-products of the transformation of the colloid into less viscous colloid C, having no direct relation to the formation of secretory products.

At any rate, such complicated secretory behavior was not found in the neurilemma, which fact may be correlated with a presumably simpler mode of hormone production in this tissue.

Recent investigations by several authors have demonstrated that the crustacean sinus gland produces various kinds of hormones in addition to the generally admitted chromatophorotropic one, such as that acting on the motility of the heart, the hormone inhibiting molting, the diabetogenic principle, the one inhibiting ovarian growth, the hormone controlling retinal pigment migration and that controlling general motor activity. With the exception of the chromatophorotropic hormone, all of these principles seem to originate in the sinus gland exclusively (*cf.* Brown, 1944; Hanström, 1947a). While it remains to be seen whether such hormones are different substances or represent only one or a few substances, it should be taken into account that there is no concrete information concerning the capacity of the neurilemma for such manifold production of hormones. It may tentatively be proposed that the observed simplicity of the secretory behavior in this tissue appears to be correlated with a restricted productivity of hormonal substances.

With respect to the histological elements responsible for the production of another kind of chromatophorotropic hormone, N hormone, experimental results have indicated that such might be located in the adult crabs in the brain and the medulla terminalis. Such a restriction in the distribution of this hormone points to incretory elements specifically present within the said two nervous organs as the possible sources. Therefore, the neurilemma universally associating with the nervous tissues is to be excluded. A marked parallelism exists between the distribution of the neurosecretory cells of the γ type and the experimentally ascertained sites of origin of N hormone.

As for the other types of neurosecretory cells, there is little possibility of their participation in the production of N hormone. This might be deduced from the experimental results demonstrating the absence of the hormone in concentrated extracts of such ganglia as the thoracic ganglion, in which the cells of the α type are present in greatest abundance, and the commissural ganglion containing the β type cells. Absence of the hormone was also demonstrated in the extract of the isolated cluster of β neurosecretory cells of the medulla terminalis.

It is of much interest that, as demonstrated above, neurosecretion of the γ type is characterized by a kind of nuclear secretory activity, which has also been reported in certain neurosecretory cells as well as in certain hypophyseal cells in some vertebrates (Scharrer, 1934; Scharrer and Scharrer, 1945; Hanström, 1947b). Thus, the production of N hormone can be regarded as being in principle identical with that of S hormone, inasmuch as both involve nuclear activity, even though the mode of nuclear behavior differs. S hormone appears to take its origin at the

expense of the nucleus, whereas N hormone results from a reversible change of intra-nuclear elements, during which the nuclear integrity remains intact.

Thus, the chromatophorotropic hormones in *Sesarma* are elaborated by two kinds of nuclear secretion, an observation which contributes further to the recently increasing knowledge regarding the participation of the nucleus in secretory activities in both the vertebrates and the invertebrates. As Palay (1943) has supported the concept of nuclear secretion against the former negative view of Bowen (1929), present work also demonstrates it in certain secretory activities.

Concerning the neurosecretion of the α and β types, no information about their physiological significance is available at present. Of interest is the role played by the secretory material of the β neurosecretory cells, especially those supplying nerve-fibers to the sinus gland. As reported above, the anilin blue granules interpreted as the final product of secretory activity of the cells were observed to take their way along the path of the axon toward the sinus gland, a fact which is reminiscent of similar figures demonstrated by Scharrer and Scharrer (1944) and Palay (1945) in the preoptico-hypophyseal tract in some vertebrates and also in the intercerebralis-cardiacum-allatum pathway in some insects. It appears plausible that the neurosecretion in question may play a significant role in the control of the secretory activity of the sinus gland. However, the present study is short of data for such considerations, except for the following. In an experiment, the nerve-cell cluster in question was carefully isolated from the medulla terminalis and extracted to be injected into eyestalk-less specimens of juvenile *Sesarma haematocheir* and eyestalk-less *Paratya compressa*, with the result that a considerable effect of S hormone (pigment concentration in reddish chromatophores in both recipients; *cf.* Enami, 1951), but not of N hormone was produced. However, whether this result is attributable to a temporary promotion of secretory activity in the recipients' sinus gland brought about by the secretory product contained in the extract, or whether it is due to the direct effect of the donor's S hormone derived from the neurilemma around the nerve-cell cluster in question, is left undetermined.

Summary

1. In this part of my contemplated series of papers are reported some results concerning the histological investigation of the incretory elements responsible for the production of the two chromatophorotropic hormones in *Sesarma*, whose activities and distribution were experimentally dealt with in the preceding study.

2. On the basis of the present histological findings, and their comparison with the experimental data, it has been concluded that the sinus gland and the neurilemma are responsible for the secretion of S hormone, whereas N hormone has its origin in a type of neurosecretory cell (γ neurosecretory cell) occurring in abundance in both the brain and the medulla terminalis.

3. The secretory activity in all of these hormone sources is alike in that it shows nuclear secretion, but there are differences in the secretory behavior.

4. The close relationship between the sinus gland and the neurilemma was substantiated on the basis of their fundamentally similar histological structure and their secretory behavior. However, certain differences were observed as to the fate of the secretory material derived from the nucleus, a more complicated sequence of transformations taking place in the sinus gland than in the neurilemma. The sig-

nificance of these differences and the possible nature of the final secretory material of the sinus gland were discussed.

5. Besides the cells regarded as possible sources of N hormone, two kinds of neurosecretory cells (α and β neurosecretory cells) were described, which are located in areas of the nervous system more or less distant from the area of distribution of the γ cells. These show a cytoplasmic secretory activity with particulate elements as their ultimate products. The physiological significance of their respective secretions is still unknown.

6. The sinus gland is innervated by a cluster of giant β neurosecretory cells of the medulla terminalis. Figures show a distal flow of secretory granules from the cells along the course of the sinus-gland nerve, a fact which is indicative of an interesting type of chemical transmission resembling those reported to occur in the innervating systems of the vertebrate hypophysis and of the insect cardiacum-allatum complex.

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CHLORIDE ION REGULATION AND OXYGEN CONSUMPTION IN THE CRAB OCYPODE ALBICANS (BOSQ)

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There are a number of species of brachyuran crabs which are terrestrial or semi-terrestrial, in contrast to the majority which are strictly aquatic. One of the relatively successful terrestrial crabs of the Atlantic Coast beaches is the "ghost" crab, *Ocypode albicans* (synonyms *O. arenaria*, Say and *O. quadrata*, Fab.). Ocypode burrows in sandy beaches, near or above the high tide level. On the Delaware beaches, where we collected it, the crab is nocturnal and spends the day in its burrow. At night it runs about the beach hunting food, and during this time it may go into the surf briefly. When alarmed, the crab runs for its burrow in preference to the sea. The incoming tide sometimes traps it in its burrow and forms a sand plug in the opening. When the tide recedes, the crab removes this plug and the burrow is useful again. So Ocypode is a crab which successfully lives in a terrestrial habitat, although this habitat borders very closely upon the sea. The anatomical adaptations of this crab have been described by Cowles (1908) and Pearse (1928).

Some brachyurans show a considerable degree of the regulatory ability which results in the maintenance of a steady internal osmotic state regardless of the fluctuations in the external environment. The degree of osmotic regulation of several species of crabs has been described by Jones (1941). He found a direct correlation between the degree to which the regulatory ability was developed and the success the crabs had shown in meeting the conditions of terrestrial life, or life where drying was a constant environmental factor. In his series Uca was the most nearly homoiosmotic. If this correlation can be projected to Ocypode, which belongs to the same family, but is more terrestrial in its habits, Ocypode also should show a high degree of osmotic regulation.

The specific mechanism of water and ion control in Ocypode has not been described. However, it is clear from the literature (Krogh, 1939) that the principle structures concerned in the crustacea are the antennal glands (kidneys) and the gills. The regulatory mechanism certainly operates at these barriers between the internal and external environments; in Ocypode it is not probable that the integument is very permeable to water and electrolytes. The regulation of water and ion movement through a membrane against a gradient is not a passive activity, but requires an output of energy during the process. Schlieper (1929) proposed the theory that homoiosmotic forms respire more rapidly in dilute than in normal sea water, while this is not the case with the poikilosmotic forms, and he suggested that energy expended by the former in resisting osmotic inflow of water is derived from some oxidative mechanism. On this basis it would be expected that any work done by a membrane upon exposure to a changed external environment would be reflected in the oxygen consumption of the animal.

The investigations reported here were concerned with the following aspects of the problem of regulation: first, the degree to which Ocypode carries on chloride ion regulation; second, the role of the antennal gland in chloride ion regulation; and third, a measurement of the oxygen consumption during the early stages of this regulation.

Methods

Crabs were collected at Rehoboth Beach, Delaware, placed in separate containers and brought back to the laboratory within a few hours of capture. They were kept in individual battery jars in which there was enough beach sand to allow burrow digging. This sand was kept moist but not soaking wet. The jars were covered to keep out direct light, and to prevent the escape of the animals. The crabs were fed all the fresh fish they would eat each night and allowed to swim in sea water for about five minutes. The once-a-day feeding and bathing routine paralleled natural conditions as we observed them and permitted us to keep the crabs in good condition for ten days or longer. Crabs had been in the laboratory for at least twenty-four hours before any work was done with them.

The degree of regulation of a single ion, the chloride ion, was studied. It is not presumed that a study of this ion alone gives a complete picture of the shifts in the internal environment. However it may be used as an indicator of the direction and degree of internal regulation.

The chloride ion concentration in the blood from individual crabs was determined. Blood was drawn from the sinus within the proximal joint of one of the legs, using a No. 27 needle fitted on a clean, dry one-quarter cubic centimeter tuberculin syringe. The usual amount of blood drawn at any time was about 100 cubic millimeters. No anticoagulant was used. It was found that quick smooth handling of the blood could effect the transfer from crab to analytical tube before clotting commenced. After the blood sample was drawn, the needle was removed from the syringe, a twenty cubic millimeter Sahli type haemoglobin pipette held to the nozzle of the syringe, and blood measured into the pipette to the calibration mark. The sample was blown into a small test tube, and the pipette rinsed into the test tube with distilled water. Samples were analyzed in duplicate, using a modification of the Volhard method. Silver nitrate was made up to 0.2906 per cent in nitric acid with ferric ammonium sulphate added as an indicator. An excess of this reagent, usually one cubic centimeter, was added to the blood sample in the small test tube. Potassium sulfocyanate solution, made up to one-half the strength of the silver reagent, was used to titrate the excess silver. Reagents were added with the syringe burette described by Scholander (1947).

Urine samples were collected by applying suction to the opercula which close the external openings of the antennal glands of Ocypode. A small vial, capacity approximately two cubic centimeters, was fitted with a rubber stopper through which passed two No. 18 syringe needles. One of these was connected by pressure tubing to a vacuum line. The cuff of the other was removed, and replaced by a short length of soft rubber tubing which could be fitted closely over the operculum. Urine collected in this way was analyzed for chloride ion content by the method used for blood.

The chloride ion present in sea water used was determined by the same method. Sea water taken at the beach from which the crabs were collected was brought back

to the laboratory, filtered and used in the experiments. It contained 480 millimoles of chloride per liter, which conforms to the figure for normal sea water used by Cole (1940). The value is lower than that determined for some open sea samples. This fact may be accounted for considering that Rehoboth Beach, Delaware, is but a few miles south of the mouth of the Delaware River. Distilled water was used to obtain dilutions amounting to 360, 240, and 120 millimoles of chloride per liter. Evaporation in shallow pans under a fan at room temperature resulted in concentrations of 600 and 720 millimoles of chloride per liter. In addition to the sea water concentrations, some crabs were put into distilled water, which is recorded as 0 millimoles of chloride per liter. Others were not put into any of the experimental solutions but remained on damp sand and are recorded as "dry."

The oxygen consumption of the crabs was measured in a respirometer of the direct reading volumetric type, similar in principle to the micro-respirometer of Scholander (1942). The apparatus is shown in Figure 1. The animal chamber

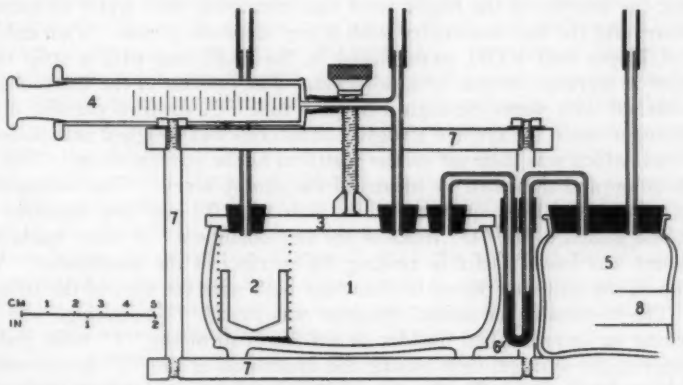

FIGURE 1. A volumetric respirometer (for explanation see text).

(1) was constructed from a finger bowl, the rim of which was ground flat and smooth. An alkali cup (2) made by drilling out a piece of one inch plexiglass rod was fastened to the bottom of the bowl with DeKhotinsky cement. A shield of plastic screen (Lumenite) placed around the cup prevented the animal from getting into the alkali. The lid of the animal chamber (3) was a disc of quarter-inch plexiglass, recessed at the periphery to close on the ground rim of the finger bowl. Three holes were drilled in the disc and fitted with rubber stoppers as shown in the figure. A three-inch length of glass tubing through one of the peripheral stoppers served as a by-pass through which fluids were added to the chamber during an experiment. A "T" tube through the central stopper had a short arm to which a ten cubic centimeter syringe (4) was attached. A small "U" tube passing through the third stopper was connected to the manometer.

The thermobarometer (5) was made from a wide-mouth specimen jar fitted with a two-hole rubber stopper. A by-pass tube through one hole opened the

chamber to the outside air. A small "U" tube connected the thermobarometer to the manometer. The manometer (6) was a tight "U" about two inches long. All glass connections in the unit were made from two millimeter bore glass tubing. Rubber junctions and extensions on the by-pass tube were made from number fourteen rubber catheter. Light weight hose clamps (not shown) were used to close the extensions.

The lid of the animal chamber was closed onto the finger bowl by a stirrup clamp (7). A four by six inch plastic platform was drilled to take two uprights made from quarter inch brass rod. The upper cross member of the clamp was a strip of plastic drilled to pass the threads of the uprights. The center point of this strip was drilled and tapped to take the thread of a thumb screw carrying a knurled knob on one end and a freely turning caster on the end bearing on the lid. The finger bowl rested on the platform. The syringe was fastened to the cross member by heavy rubber bands. The thermobarometer was fastened to one of the uprights with a half inch metal strap (8).

In use the interior of the finger bowl was moistened with water to saturate the atmosphere, and the rim was coated with heavy stopcock grease. Two cubic centimeters of 10 per cent KOH were placed in the alkali cup with a strip of folded filter paper to increase surface for absorption. The interior of the thermobarometer was moistened with water through a by-pass tube. A drop of paraffin oil spread on the plunger made the syringe airtight. The crab was weighed and placed in the finger bowl, which was then set on the platform of the stirrup clamp. The lid was put in position and tightened by means of the thumb screw. The manometer was filled to a height of three-quarters of an inch with 0.1 per cent aqueous tergitol solution and joined to the "U" tubes of the two chambers. A rider made of white plastic sheet was found useful in reading the menisci of the manometer. With all outlets open, the unit was placed in the water bath with the tops of the tubes out of water. The by-pass of the animal chamber was closed. The syringe was charged by attaching an oxygen-filled bladder to the free end of the "T" tube, pulling out the plunger of the syringe, then closing the upper end of the "T" to prevent loss of oxygen from the system. The by-pass of the thermobarometer remained open during the equilibration period of twenty minutes. The syringe was constantly adjusted inward to keep the menisci of the manometer level. At the end of the equilibration period the thermobarometer by-pass was closed, and the initial reading of the syringe recorded. During the course of the experiment oxygen consumed was replaced from the syringe, and the syringe readings gave a direct measure of the oxygen consumption of the animal. The respirometry was done during the summer at a room temperature of 26° C.

The oxygen consumption of the crabs which are described as "dry" in the respiration results was measured in this way for a period of an hour and a half. Regassing the syringe during this time was done without removing the apparatus from the water bath, by attaching a bladder to the "T" tube. Added oxygen was in thermal equilibrium with the water bath. Crabs which were tested for response to a new environment were treated in the following way. The oxygen consumption was determined for thirty minutes, with the crabs in a moist chamber as described. At the end of thirty minutes, the taps were opened, and one hundred cubic centimeters of water of a measured salinity were introduced into the respirometer

chamber through the by-pass tube. This water had been well shaken with air, and had been allowed to equilibrate to the temperature of the water bath. The taps were closed and oxygen consumption of the same crab measured for a full hour. In this way, the sequence of oxygen consumption in air and in environmental fluids of various salinities could be measured.

Blank runs, with the animal chamber moist and with the introduction of water were made following the exact experimental procedure except that no animal was used. The results showed no measurable drift for two hours following equilibration.

Results

Ocypode spends most of its time out of water, either in burrows or running on the sand. The blood chlorides of crabs from sand were determined as a normal value. The mean of sixty-four "dry" determinations was 378 mM Cl/L, with a standard deviation of 24.27. This indicates that two-thirds of the data falls between the values of 354 and 402 mM Cl/L, a spread of 12.7 per cent. The mean is well below the figure for normal sea water, which was 480 mM Cl/L, being much nearer 75 per cent sea water, or 360 mM Cl/L. In view of the spread of the "dry" determinations, it was thought more valuable to compare the reaction of a crab to a new environment with its own "dry" chloride level rather than with a mean of that group or of a control group. The experimental procedure was, then, to determine the chloride of a crab on sand, expose the crab to the experimental sea water, and determine the blood chloride again at specific time intervals. The ratios of the experimental to dry values were calculated. The mean of the ratios for three to six individuals in each environmental situation after one, three, and twenty-four hours are plotted in Figure 2, with the standard deviations in each case represented by a vertical line. One group of crabs remained on sand; the blood chloride was determined for the same time intervals, and treated in the same way. The ratios are recorded in Figure 2 as "dry."

There are no significant changes in the blood chloride levels of individual animals exposed to the various environments for one or three hours. The blood chloride of Ocypode remains steady for a period of twenty-four hours in air, and under the conditions of exposure to sea water diluted to 120 and 360 mM Cl/L and evaporated sea water containing 600 mM Cl/L. There is some difference in the ability of individual crabs to maintain a steady internal chloride level, but the fluctuations observed are not statistically significant. When the crabs are exposed to distilled water or to water containing 720 mM Cl/L for twenty-four hours, the internal chloride does not remain steady, but tends to fall in distilled water and to rise in the high concentration of sea water. Within twenty-four hours there is a significant change in the internal chloride in these extreme ranges although these values do not yet equal those of the external environment. It was found difficult to carry the crabs beyond this time, or to restore them in normal sea water, although they survived three days or longer in all the other environments.

Urine was collected from crabs on sand and from crabs which had been in experimental solutions. An attempt was made to collect urine at one hour, three hour and twenty-four periods, but it was found that the shorter intervals were unsatisfactory. No attempt was made to determine the volume of urine. Ocypode

often ejects the urine when handled, and many samples were lost in this way. Crabs from sand were most often lacking in available urine but it was usually obtained after they had been in any of the solutions for twenty-four hours. The urine chlorides of crabs on sand were determined. The mean of eleven "dry" determinations was 455 mM Cl/L, with a standard deviation of 46. The mean value falls above that for blood, and below that for normal sea water. The results of urine chloride determinations at the twenty-four hour period were treated in the same way as those of the blood chlorides, and are plotted in Figure 3. The mean ratio

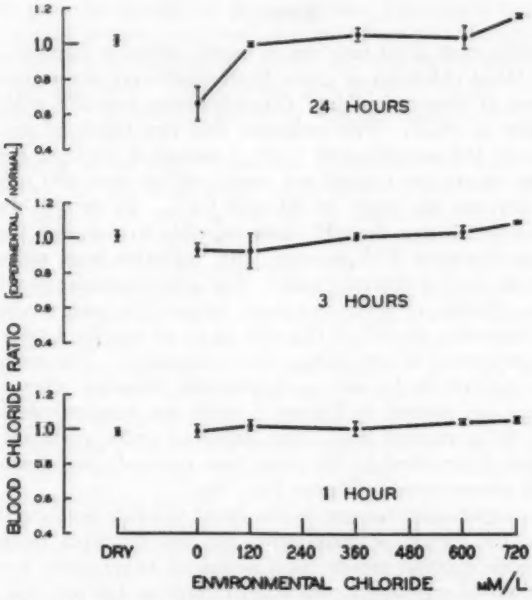

FIGURE 2. Relationship between blood chloride and environmental chloride concentrations.

of urine chloride of three crabs in each environment is plotted, and these points are connected by a solid line. The standard deviation in each case is indicated by the vertical line. The urine chloride ion concentration varies in the same direction as the concentration of the environment, being low in dilute solutions, high in concentrated solutions. There is a wider deviation in the results in the extremely dilute and extremely concentrated environments than there is towards the more nearly isotonic environments.

To determine the effect of the regulatory work on the oxygen consumption, the respiration of crabs from sand, and of crabs in sea water of a range of salinities was measured. The oxygen consumption of six individuals from sand was determined on each of three consecutive days. The mean and the standard deviation were calculated for each of the six individuals, on the basis of data from three con-

secutive daily determinations. The six means and standard deviations so determined were averaged to give the average mean and average standard deviation of six individuals. The mean oxygen consumption was 0.139 cubic centimeters of oxygen per gram fresh weight per hour, with a standard deviation of 0.019. This indicates that about two-thirds of the data fall in the range 0.120 to 0.158 cubic centimeters of oxygen per gram per hour. This treatment gives a more representative picture than would (1) the standard deviation of all eighteen determinations, which would deny that there were six sets of repeat determinations; or (2) the standard deviation of the six means of three determinations each, which would mask the fluctuations within a series of determinations on each crab. It is apparent that the conventional use of a volume of data as the "normal" or control is impossible in the face of such wide deviations. It was decided, therefore, to compare the response

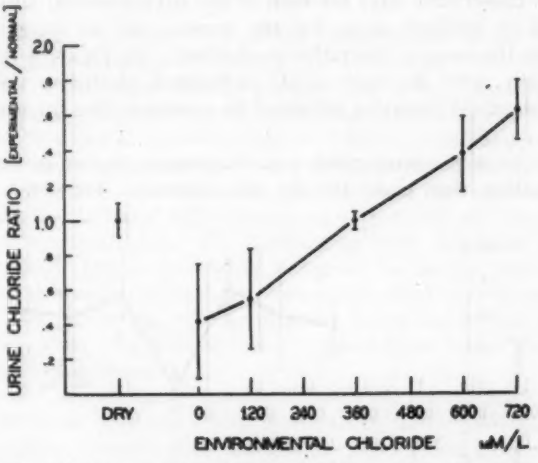

FIGURE 3. Relationship between urine chloride and environmental chloride concentrations after twenty-four hours.

of a single crab to a change in the external environment with the dry oxygen consumption of the same crab. This conformed to the treatment of the chloride data.

In the respiration measurements, the oxygen consumption of the crabs was determined the first thirty minutes with no standing water in the moist chamber, hence the animals could be termed "dry". This thirty minute period is designated "A". The oxygen consumption was determined for one hour after the experimental sea water was added to the respirometer. The first thirty minutes after the addition is designated "B", and the second thirty minutes "C". It could be predicted that if the oxygen consumption rate did not change, but a steady rate was maintained during the entire ninety minutes, period "B" would equal "A", and periods "B" plus "C" would equal twice "A". A graph of this steady state would be a straight line.

TABLE I

Environment in mM Cl/L	"B"/"A"	"B" + "C"/"A"
0	1.50	2.53
120	1.54	2.59
240	1.58	2.73
360	1.15	2.17
480	1.41	2.69
600	1.38	2.57
720	1.35	2.61
"Dry" (normal)	0.95	1.84
predicted ratio	1.00	2.00

The data on oxygen consumption were calculated as ratios of the period "B" to period "A", and of period "B" plus "C" to period "A". Six individuals were tested on three consecutive days for each of the environmental situations, and the data calculated as outlined above for the normal rate of oxygen consumption. Table I presents the mean of the ratios so obtained. In Figure 4 the data are presented graphically, with the mean of six individuals plotted as the central point, and the mean standard deviation indicated by a vertical line for each set of determinations.

The rate of oxygen consumption was consistently higher in water, regardless of the concentration, than under the dry circumstance. This cannot be attributed

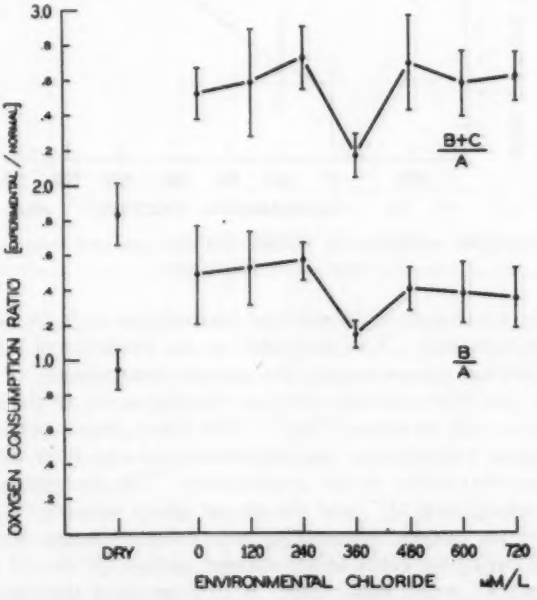

FIGURE 4. Oxygen consumption related to environmental chloride.

to diffusion of gas into the introduced water or oxygen consumption by organisms in the water. Check runs complete except for the crab failed consistently to give discernible evidence of any but a steady state of the respirometer. The rate of oxygen consumption in water containing 360 millimoles of chloride per liter was significantly less than the rate in any other salinity. This concentration most closely approximated the normal internal chloride concentration of the crab, which was determined as 378 millimoles of chloride per liter. In concentrations of sea water greater than or less than this nearly isotonic concentration, the rate of oxygen consumption increased significantly. The increase in dilute solutions was greater than in hypertonic solutions. There was no evidence of progressive increase as the extremes of salinities were approached. There was, however, a wider fluctuation in individual results, particularly in the dilute series.

Discussion

Ocypode maintains a steady chloride ion concentration in the blood in air and against a wide range of external chloride concentrations. The mean blood concentration is 378 millimoles of chloride per liter, and is held steady against external concentrations ranging from 120 to 600 millimoles of chloride per liter. When the crab is exposed to external concentrations of 0 and 720 millimoles of chloride per liter the steady state is not maintained and a drift towards the lower or higher value is observed. At the end of twenty-four hours in these two extremes, the chloride ion concentration in the blood falls as much as 40 per cent or gains as much as 15 per cent over the normal level. No observations were obtainable on crabs which had been exposed to the environmental extremes for several days. It was very difficult to keep them alive beyond the twenty-four hour period, especially in distilled water. The decreasing viability probably is not due to the shift in chloride ion concentration alone, but is indicative of a breakdown in the mechanisms regulating water and ion transport.

The ability of Ocypode to regulate the chloride ion concentration of the blood is in agreement with the observed ability of the more terrestrial crabs, Uca, in particular, to approach a homoiosmotic condition. Jones (1941) has shown that Uca regulates against both hypotonic and hypertonic environments. The fact that the mean value of blood chloride for Ocypode is well below the figure for sea water, which was 480 millimoles of chloride per liter at the site where the crabs were taken, indicates that the crabs which made the transition from sea to land possess the mechanism fundamental also to those which move from sea to brackish water. According to the hypothesis proposed by Beadle and Cragg (1940), a contributing factor to successful transition from sea to brackish or fresh water is the maintenance of a chloride ion concentration in the blood somewhat lower than that of sea water, hence, presumably easier for the animal to maintain against the brackish water gradient. Other members of the family Ocypodidae, Uca for example, invade brackish water as well as spending much time in air. The lower internal chloride concentration of Ocypode is obviously easier to maintain in the terrestrial habitat wherein food and infrequent contact with sea water are the only sources of chloride ions.

The maintenance of a steady internal chloride ion concentration is accomplished by regulation of water intake and loss, and salt intake and loss. The data we have

collected on urine chloride concentrations indicate that the antennal gland of Ocypode functions in chloride ion control. If the chloride ion content of blood and urine from crabs on sand, the "dry" or "normal" values, are employed with the ratios shown in Figures 2 and 3, and the representative levels of blood and urine chloride under the imposed environmental conditions are calculated, a graph such as Figure 5 may be made. Calculated levels for urine chloride are indicated by a solid line,

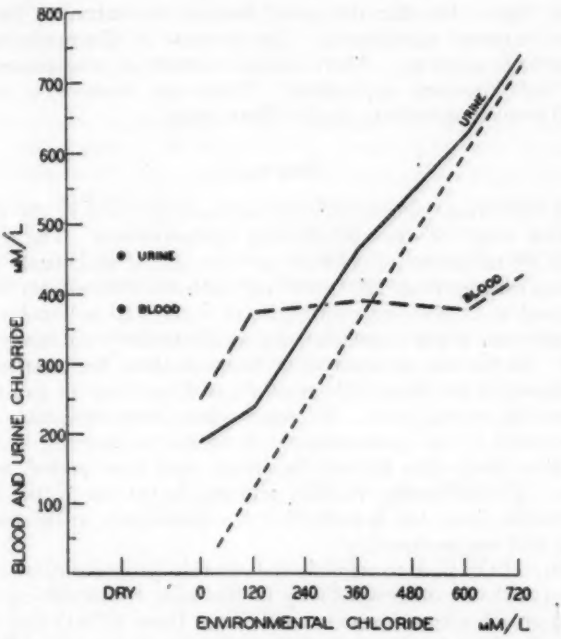

FIGURE 5. Calculated blood and urine chloride concentrations related to environmental chloride.

for blood by a broken line, and for the environmental fluid by a dotted line. The line representing the environmental fluid crosses the line for blood chloride at the isotonic level, about 380 millimoles of chloride per liter. In the situation of the chloride ion concentration in the external environment being equal to that in the internal environment, no accumulation of chloride in the urine would be expected. However, as the data indicate, chloride ion is present in the urine in excess at the isotonic point. This is probably due to the re-absorption of water by the antennal gland. Supporting evidence for this conclusion may be drawn from two observations: first, the urine chloride ion content when the crab is in a medium containing 360 mM Cl/L is not significantly different from the urine chloride concentration of crabs on sand. Water saving by re-absorption is important for a terrestrial animal, and it would appear to be the activity reflected here. Second, the oxygen consumption of crabs in a medium containing 360 mM Cl/L is the lowest of all the rates of

crabs in fluid environments, indicating a minimum of regulatory work being done.

The chloride ion concentration in the urine varies in the same direction as that in the environment. However, the urine chloride concentration is equal to the blood concentration only when the crab is in an envionment containing about 270 mM Cl/L. In sea water containing more chloride than this value, the urine tends to contain more chloride ion than the blood; in more dilute media the concentration of chloride ion in the urine is less than in the blood. Here is indication that the antennal gland is active in the control of the chloride ion concentration in the internal medium, either actively secreting chloride or re-absorbing water, over a considerable range of external environmental salinity. This is an expected activity when the chloride ion concentration in the environment is higher than that in the blood. Further examination of the data shows that in very dilute solutions the antennal gland wastes chloride, producing a urine that has a high chloride ion content as compared with the external environment. The fact that a high urine chloride loss persists in distilled water, despite the fact that the blood chloride level is falling, may be interpreted to mean that the antennal gland does not have a critical threshold for the chloride ion.

From Figure 5 it may be seen that at the 270 mM Cl/L level in the external environment, blood and urine chloride concentrations are equal. Animals in this range of environmental chloride survived for several days and maintained a normal and steady blood chloride level. It is clear that at this point proportionately more chloride than water must be obtained from the external environment. The fact that the blood chloride level is maintained steady in hypotonic environments in spite of an overactive excretion of chloride by the antennal gland is further evidence that some chloride-absorbing mechanism is at work. It is assumed that the mechanism here is selective absorption of chloride ion by the gills. Webb (1940) has shown that the gills of Carcinus do actively absorb chloride ion from the external medium. He has further shown that there seems to be no reason to assume an irreciprocal permeability of the gut and integument. It might be pointed out that the integument of Ocypode does not appear to permit evaporation of water when the animal is on land, and it is assumed that it would not permit great exchanges of salt and water when the crab was immersed. We have not yet completed studies as to the possibility of specific tissues being reservoirs of internal salts and water.

It is seen from the data on oxygen consumption of crabs from sand and from environments of different salinities that work is being done to maintain the internal chloride ion concentration, and, it may be projected, the internal osmotic state. The membranes involved, gill and antennal gland, although affording ample surface for the exchange of materials, do not make up a large part of the mass of the animal. In order to determine the metabolic reaction to changing environments it is necessary to use an intact animal, and to measure a change dependent on the activity of a very small per cent of the weight of the animal. It was felt that with the combination of measuring a small overall change and the inherent variation between individuals, statistical treatment of the data would prevent overlooking pertinent relationships.

In order to relate the data obtained on oxygen consumption to the observed regulation of the internal chloride ion concentration, the ratios of the first experimental period to the control period ("B"/"A") are represented in Figure 6 in block

form. If the oxygen consumption in air (solid segment) is assumed to represent the normal or control value, and if this amount is then removed from the values for oxygen consumption in water, the increment in water must be due to work, either of a mechanical or osmotic nature. The increment is least, about 21 per cent, in an external environment of 360 mM Cl/L. From Figure 5 it is seen that at this concentration the blood and external chloride concentrations are nearly equal. As suggested earlier, at this environmental level, the gill is probably doing little or no osmotic work, since no gradient exists between internal and external environments. On the basis of the urine chloride data, the antennal gland work at this level is no more than is being done by the "dry" crab on sand. Therefore, most, if not all of the increment in oxygen consumption at 360 mM Cl/L is due to

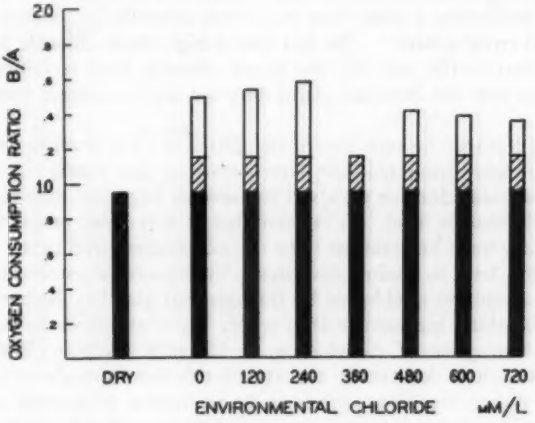

FIGURE 6. Oxygen consumption related to environmental chloride to show partition of work between air and water ventilation and between water with and without chloride gradient.

mechanical or muscular activity. In view of the greater mass and viscosity of water it is reasonable to assume that water bailing requires more energy than air ventilation. Ocypode has reduced gills and branchial tufts according to Pearse (1928). In air the diffusion of oxygen through these respiratory surfaces is sufficient for the requirements of the animal, probably with no more accessory ventilation than is accomplished during leg movements. In the respirometers the crabs showed a definite thigmotactic response and were quiet during the time of the experiment. When Ocypode is placed in water, a scaphognathite attached to the second maxilla sets up a rapid beat which serves to force water from the posterior branchial chamber opening between the bases of the third and fourth legs through the branchial chamber and out at the mouth region. Two "gill rakers" attached to the first and third maxillipeds also beat rapidly when the crab is in water. This constant muscular activity which is not present in crabs in air may account for the increase in oxygen consumption in environmental fluid containing 360 mM Cl/L.

A possibility which merits further investigation is that some of the increase noted when the animals were placed in water may be due to the payment of an oxygen

debt incurred as a result of drying of a significant portion of the respiratory surface, or as a result of inadequate ventilation in air when the animal is quiet. In an environment containing 360 mM Cl/L, the ratio of respiration in the first experimental period to the control period is 21 per cent higher than the same ratio of crabs "dry" throughout the experiment. The ratio for the second experimental period is only 14 per cent higher than the same corresponding ratios for "drys." Measurement of the respiration was not continued past this point. Not enough is known of the respiration pattern of Ocypode to make a definite statement concerning the possibility that these increments indicate payment of oxygen debt. However, two observations may be considered here: (1) crabs on moist sand ten days are just as active and maintain the activity as well as crabs exposed to periodic wetting, which would indicate no cumulative oxygen debt; and (2) the pattern of decrease in the second period does not change with increasing time of exposure to air. The possibility of early saturation of the oxygen debt capacity complicates this latter observation. It may be speculated that if there is an oxygen debt present in Ocypode in air, this represents a phase of terrestrial adaptation which has not yet become perfected in this species.

The increase in oxygen consumption in 360 mM Cl/L as compared with that in air is represented in Figure 6 by the cross hatched segment. If this portion, plus that corresponding to the respiration in air, is then removed from the total block for each environment, the remaining clear segment may be assumed to represent the osmotic work done in maintaining a steady internal environment against a gradient. In hypotonic solutions the increase attributable to this regulatory work is of the order of 34 per cent, decreasing slightly in the more dilute environments. This increase represents the sum of the work of two regulating mechanisms. The gill membrane is working to absorb chloride ion from the environment, and is allowing water to pass into the crab, while the antennal gland is bailing water out again. In the more dilute environments the antennal gland appears to be inefficient. In Figure 5 it is seen that the amount of chloride excreted is in excess of what would be predicted on a straight clearance basis, which would indicate that the antennal gland is failing to re-absorb chloride ion or is not bailing the water necessary to maintain the balance. Webb (1940) has suggested that in very dilute solutions the gill of Carcinus becomes less permeable to water. The data presented here seem to be in accord with this suggestion. The gill continues to absorb chloride ion from the environment, while allowing less water in proportion to pass the barrier; the antennal gland then clears less water, with less energy expenditure than would be expected. The two mechanisms are able to maintain an internal chloride ion level in solutions containing 120 millimoles of chloride per liter or more. No points intermediate between this dilution and 0 were tested, so that the exact limit to which the regulation is maintained is not known.

In solutions containing more than 360 mM Cl/L there is an increase in the rate of respiration of the order of 20 per cent. In hypertonic solutions the gill ceases the selective absorption of chloride ion from the environment, and as far as we know, has no chloride-secreting mechanism. However, chloride ion and water pass through the gill membrane in accordance with the Donnan equilibrium. The antennal gland is the chief mechanism of regulation in hypertonic environments, excreting chloride ion or saving water, or both. The antennal gland working alone

does not require as much oxygen as gill and antennal gland together do in hypotonic solutions. In environments of high concentration, 720 mM Cl/L, the ability of the antennal gland reaches a limit and cannot keep up with the incoming chloride ion. Hence, the blood chloride ion level rises. The upper limit on antennal gland ability is also shown by the fact that the oxygen consumption does not progressively increase with increase in environmental chloride. The slightly lower oxygen consumption in the greatest concentration may be the result of metabolic depression caused by the concentration of ions other than chloride.

With the hypothesis of Schlieper (1929) in mind, inspection of the oxygen consumption rate across the spectrum of osmotic insult would indicate the point at which the external and internal environments approached isotonicity. The elevation of the oxygen consumption rates on either side of this isotonic point may be accounted for, as indicated above, by the energy output of gill and antennal gland, each in a twofold role of chloride ion capture and clearance.

Further examination of the respiration and urine chloride data indicates that the deviation from the mean is much wider at the environmental extremes, than towards the isotonic level. It may be postulated that here is shown a range of response of single factor magnitude which might form a basis for selection. In the past, the antennal gland function, especially on the hypertonic side and in air, may have been an important factor in the selection of crabs as they passed from water to the terrestrial habitat.

Summary

1. *Ocypode albicans,* a brachyuran crab with a terrestrial habitat, is able to regulate the chloride ion content of the blood in the dry habitat and in sea water of salinities ranging from 120 to 600 millimoles of chloride per liter. The mean value of the chloride ion concentration in the blood is 378 millimoles of chloride per liter. This value is lower than that of sea water at the site the crabs were collected (480). It is suggested that the lower concentration of chloride is easier for the terrestrial animal to maintain in an environment where chloride is available only from food or from occasional contact with the sea.

2. In sea water containing less than 120, or more than 600 millimoles of chloride per liter, the internal concentration is not maintained, but tends to fall in dilute solutions, rise in the more concentrated ones. This internal shift is significant in a period of twenty-four hours, and proves fatal if continued for an additional twenty-four hours. The antennal gland functions in the regulation of the internal chloride ion concentration. It re-absorbs water when the crab is in air and in hypertonic solutions. It excretes water when the crab is in hypotonic solutions, and may excrete chloride also. The evidence points to another organ functioning in the reverse way; this is assumed to be the gill membrane.

3. The oxygen consumption of Ocypode in air is of the order of 0.139 cubic centimeters of oxygen per gram fresh weight per hour. In water the crab uses a gill bailer and gill rakers constantly, and the respiration rate is elevated. The increase is least when the crab is in an environment in which the chloride ion concentration equals that of the blood. In hypotonic and hypertonic environments the oxygen consumption is further increased. This is caused by the increased activity of the ion-regulating membranes of the antennal glands and gills.

4. Comparison of individual responses to the different environments indicates a difference in individual ability to meet the stress. The differences are more widely marked in environments most different from the internal medium. It is suggested that this is a measure of the variation of a physiological mechanism which was probably important in the evolution of the crab from the aquatic to the terrestrial habitat.

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PHOSPHATE UTILIZATION BY DIATOMS[1]

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Routine measurements of inorganic phosphorus concentration are frequently made on standard oceanographic stations to ascertain the potential productivity of marine waters. In quantitative, ecological studies on diatom populations in the Atlantic Ocean, Riley, Stommel and Bumpus (1949) have utilized the phosphate concentration as a nutrient index. Their equation of productivity depends upon the experimental results of Ketchum (1939), who found that phosphate concentrations below 0.55 microgram-atoms per liter limited the growth of *Nitzschia closterium*.

Riley, Stommel and Bumpus (1949) further checked the validity of this index in their photosynthetic rate equation by calculating the phosphate requirements of a growing plankton population, assuming that 100 carbon atoms to one atom of phosphorus are needed for normal metabolism. However, in studies on the fresh water diatom *Asterionella formosa*, Lund (1950) found that the phosphate content of the plant varied by a factor of seventy in both natural and cultural populations. He stated that the cells are able to store phosphorus in excess of their immediate requirements even at very low levels of phosphate concentration.

The data on the amount of phosphate contained in marine phytoplankton, as found by Ketchum and Redfield (1949), show considerable variation, making the determination of phosphate requirement difficult and uncertain. The use of phosphate as a parameter in the computation of productivity would appear to be premature therefore, until such time as definitive values of the phosphate requirement shall have been established. Even then the total mineral requirements relative to one another must be known and their seasonal and geographic abundances must be shown to vary with biological activity. The use of an arbitrary and easily measured nutrient index for correlation with diatom abundances seems to be unwarranted.

The release of phosphate by marine plankton is an important factor in ecological investigations. Cooper (1935) found that one-fourth to one-half of the phosphate added as phytoplankton in sea water was set free to the environment as inorganic phosphate after one month. Seiwell and Seiwell (1938) reported similar results.

Because of the scarcity of experimental data on the uptake of phosphate and its subsequent release from diatoms, controlled experiments using radioactive phosphate as a tracer were devised to follow the path of phosphate quantitatively from the marine environment to the plant as a function of time. This present study initiates a series of investigations which will attempt to determine by modern radiochemical techniques the minimal quantities of mineral nutrients needed for optimal growth and multiplication of a diatom species in culture.

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Laboratory Techniques

Stock cultures

Single cells or colonies of cells were isolated from inshore water samples taken at the pier of the Scripps Institution of Oceanography, La Jolla, California. Isolated cells of a given species were washed by passing through three drops of sterile nutrient sea water medium, and were then placed in 10 ml. of medium in a test tube. When the diatom population had reached approximately 1000 cells per ml., a one-ml. aliquot was transferred to a cotton-stoppered 250-ml. Erlenmeyer flask containing 100 ml. of a sterile nutrient sea water medium, as described below. Cultures were maintained by transferring one ml. of culture to a flask of fresh medium at two-week intervals. The original cells of the *Asterionella japonica* culture used in the early part of the work had been isolated from the plankton sample in the fall of 1948; the initial cells of the *Asterionella japonica* later utilized were isolated from the plankton samples in the winter of 1950.

Preparation of the nutrient sea water medium

A basic requirement in the radiochemical assay of the partition of phosphate between the nutrient medium and the diatoms is that the total phosphate be contained only within these two fractions. Thus, any phosphate precipitation during the preparation of the enriched sea water or during the course of the experiments invalidates the measurements. Microscopic examination of culture waters prepared in the normal way revealed particulate matter which was found by chemical analysis to be iron phosphate. This solid phase was able to adsorb and to contain a considerable portion of the phosphate. Since the cells often clung to, or were entrapped, or covered by these masses, these precipitates tended to obscure the diatom cells during visual counting, making the counting more difficult. Therefore, the sea water collected for use in the culture medium was subjected to special treatment before and during enrichment to prevent any formation of particulate matter.

The sea water used in the preparation of the medium was collected in glass containers, approximately 14 liters at a time, from the pier. It was filtered through either S & S No. 489 Blue Ribbon or Whatman No. 42 paper before enrichment with nutrients.

The medium used was a modification of Allen and Nelson's (1910) culture solution (Table I). The sea water and the nutrients were sterilized in separate containers. The salts were sterilized in two portions: one, a combination of the KNO_3, $MnCl_2$, and Na_2HAsO_4; the other, the phosphate made up to 100 ml. of

Table I

Culture medium nutrients

Solution		Amount of solution added per liter
M/10	KNO_3	20 ml.
M/100	$MnCl_2$	0.1 ml.
M/100	$CoCl_2$	0.1 ml.
M/100	Na_2HAsO_4	0.1 ml.
3M/1000	K_2HPO_4	Varied through investigation
M/10	Ferric citrate	0.1 ml.

solution with distilled water. The sea water was also sterilized in two portions of three liters each in 4-liter reagent bottles. Sterilization was achieved in the autoclave at 120° C. and 18 pounds pressure. Exposure times were 10 minutes for the salts and 25 minutes for the sea water. After cooling, the water and salt solutions were combined in a sterile 9-liter serum bottle, the salt solution entering through a separatory funnel as the sea water simultaneously passed through glass tubing to the bottom of the bottle. The KNO_3 combination was added with the first three liters of sea water, and the phosphate with the second portion of the sea water. The iron citrate solution was added directly to the culture bottles before diatom inoculation.

Bio-assay

Water samples sometimes failed to produce growth in spite of enrichment. To avoid cultural failures, all of the water collected was first assayed. After preparation of the enriched water, a sample was drawn and placed in several stock culture flasks and then inoculated. An initial count was made and the cultures re-counted twice at weekly intervals. Not infrequently these preliminary assays showed that, in spite of enrichment, the water was refractory to diatom growth. Such water was never used in the radiochemical experiments.

Radiochemical culture techniques

Seven hundred ml. of the enriched sea water was added to the one-liter wide-mouthed culture flasks (Fig. 1), which were stoppered with cotton plugs. The water within the culture bottles was stirred by means of a floating magnetic stirrer made from a 50-ml. round bottom boiling flask. In initial experiments in which the magnetic stirring rod was allowed to rest on the bottom of the culture bottles, the diatoms were destroyed. The solutions were stirred for a period of one minute out of every fifteen minutes with a Haydon Repeat Cycle Timer. It was found that at this stirring rate all of the diatoms received adequate light for normal growth. Continuous stirring resulted in some diatoms clinging to the walls of the glass vessel.

Six such flasks were kept in a constant temperature room at 18° ± 2° C. Five of the flasks contained diatoms while the sixth flask was used as a radiochemical control. The six culture flasks received an equal illumination from a 48-inch, forty-watt fluorescent lamp. The light intensity, as measured with a Weston Photronic Foot Candle Meter, was 55 ± 5 foot candles.

Diatoms were introduced at an inoculation level of about 400 diatoms/ml. Simultaneously, a 10-microcurie aliquot of P^{32} as phosphate ion [2] in sea water was added so that the counting rate of one ml. of the initial solution was about 3000–4000 counts per minute as measured under an end-window Geiger-Muller tube with a window thickness of 1.4 mg./cm.^{2} and a geometry of nine per cent.

Daily radiochemical assays were made on the cultures to determine the phosphate content of the total solution, the diatoms, and the liquid medium freed of diatoms by the following method: one ml. of the whole solution (after continuous stirring for five minutes) was evaporated to dryness under an infrared lamp on a 10

[2] The radioactive P^{32} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

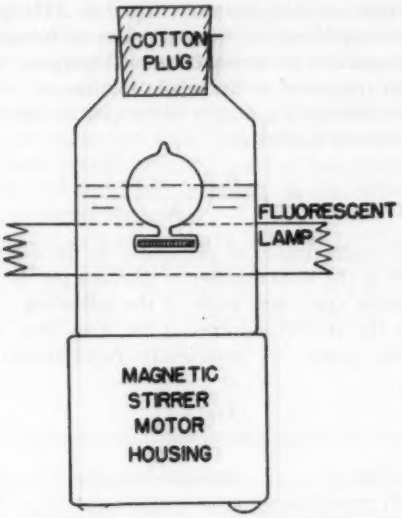

FIGURE 1. The diatom culture bottle. Six such bottles were aligned in front of a single fluorescent lamp for a single experimental run.

cm.^{2} copper planchet. When the activity is plotted as a function of time, the known decay rate of 14.3 days of P^{32} is obtained (Fig. 2). This result indicated that there was no loss of phosphate during the experiment by adsorption on the walls of the glass vessel or by the clinging of diatoms to the walls or bottom of the culture flask. It further revealed that, within experimental error, the volume of diatoms at maximum growth was small with respect to the one-ml. aliquot, otherwise a dilution of the activity with time would have been observed.

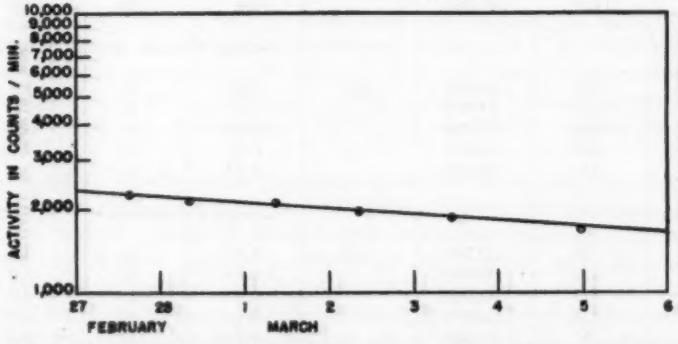

FIGURE 2. The radioactivity of the phosphate in an aliquot of the culture medium containing the diatoms is indicated by the open circles. The theoretical decay of P^{32} is shown by the heavy line. Data from experiment 3T.

Three ml. of the whole solution was centrifuged at 2000 rpm. and one ml. of the supernatant liquid was evaporated to dryness under an infrared lamp on a 10 cm.^{2} copper planchet. The activity of the supernatant liquid, a_s, was assayed under the Geiger-Muller tube and compared to the whole solution, a_t. We thus arrive at the value of phosphate per diatom, P_d, at any given time t, the time of withdrawal of the aliquots from the culture bottles as

$$P_d = \frac{P_t(1 - a_s/a_t)}{N}, \qquad (1)$$

where P_t is the initial concentration of phosphate in the culture medium in microgram atoms/ml. and N is the total number of diatoms per ml.

The diatom population assay was made in the following way. One-ml. aliquots were withdrawn from the stirred cultures at the time that the samples for radiochemical analysis were taken. A sufficiently representative population of this

TABLE II

Culture flask	N, cells/ml.		P_t, micro-gram atoms/liter		Growth constant, hours	Final P_d, microgram-atoms $\times 10^8$
	Initial	Final	Initial	Final		
1L	318	122,000	12.8	4.4	15	6.9
2L	612	48,000		4.3	15	8.9
3L	1059	68,000		8.5	17	6.3
4L	450	76,000		5.4	13	9.7
5L	625	68,000		4.5	17	6.6
				Average with std. dev.		7.7 ± 1.5
10	1760	80,000	8.4	4.2	20	5.3
20	1970	40,000		4.4	20	10.1
30	2610	62,000		4.8	20	5.7
40	1320	54,000		4.0	16	8.2
50	1140	40,000		6.4	20	4.9
				Average with std. dev.		6.8 ± 2.2
1T	783	84,000	4.2	0.59	13	4.3
2T	327	72,000		0.57	11	5.0
3T	396	75,000		0.69	11	3.3
4T	420	43,000		0.76	12	7.7
5T	321	40,000		1.22	14	7.5
				Average with std. dev.		5.6 ± 2.0
1W	66	52,000	2.7	0.25	17	3.9
2W	253	30,000		0.25	12	5.0
3W	375	41,000		0.20	12	6.0
4W	429	52,000		0.15	12	4.9
5W	267	45,000		0.22	12	5.7
				Average with std. dev.		5.1 ± 0.8

was counted to obtain the density of the population expressed as cells per ml. Care was taken that the cells counted were normal and healthy. Moribund or dead cells were not numerous except in aged cultures, *i.e.*, in some cultures made in early experiments before the bio-assays were made.

The supernatant liquid of the radiochemical control flask was assayed radiochemically, and its activity was compared with that of the whole solution. Any difference would indicate phosphate precipitation. In all of the experiments the activity of the supernatant liquid and the whole solution of the radiochemical control flask were the same within experimental error.

The phosphate analyses were made by the Wooster and Rakestraw (1951) modification of the method of Deniges-Atkins on the solution before the addition of the radioactive phosphate solution. The maximum concentration of phosphate introduced by this addition was 0.01 micro-gram atoms/liter. The phosphate remaining in solution, P_s, at any time t is given by

$$P_s = (a_s/a_t)P_t. \tag{2}$$

Results

Diatom culture experiments were carried out in media varying in initial phosphate levels from 2.7 to 12.8 microgram-atoms per liter. Table II shows the results found between the diatom populations and the phosphate in solution. Columns 2 and 3 represent the initial and final cell count of the diatoms, the final count representing the population when the first signs of leveling-off appeared in the cultures. It should be noted in column 3 that essentially the same final concentrations of diatoms were obtained in all runs. Columns 4 and 5 represent the initial and leveling-off values of the phosphate remaining in solution. The growth constant, or the time for a given cell to divide, is given in column 6. The final concentration of phosphate per diatom as calculated from equation (1) is given in column 7. It is seen that the final phosphate content of the diatoms and the maximum cell population are essentially independent of either the initial inoculum or the initial phosphate level. The spread in the figures is undoubtedly due to cumulative errors in counting of the diatoms, radiochemical assay and sampling techniques.

If one takes as the phosphate content per diatom 5.0×10^{-8} microgram-atoms per cell and a population of 50,000 cells/ml., it is seen that 2.5 microgram-atoms of phosphate per liter are needed for a further division. In experiments T and W there was not enough phosphate remaining in solution to support much further growth.

The dynamic aspects of the phosphate uptake by diatoms are presented in Figures 3 and 4. Figure 3 depicts the relationship between the phosphate remaining in solution and the population level during an experimental run. It is seen that the point at which the diatoms reached their peak growth coincides closely with the leveling-off of the phosphate uptake at 0.25 microgram-atoms per liter. Evident in Table II, in the four replicate culture flasks of run W, is the leveling-off of the phosphate uptake and diatom growth which occurred at phosphate concentrations in the solution between 0.15 and 0.25 micro-gram atoms of phosphate-phosphorus per liter. It was further found that after the leveling-off period, the phos-

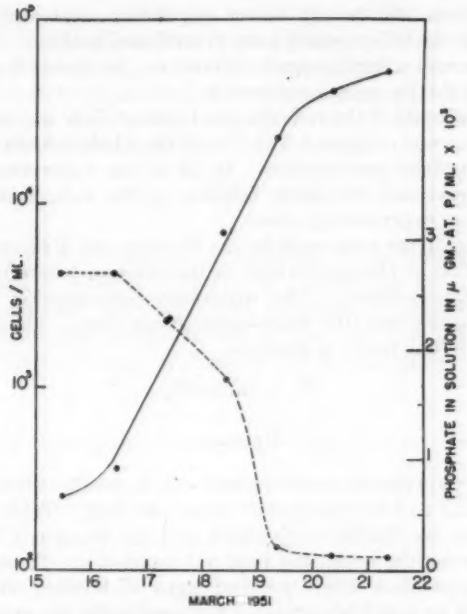

FIGURE 3. Phosphate depletion from solution in an actively growing culture. The heavy curve with solid points represents the exponential growth of diatoms, while the broken curve depicts the phosphate remaining in solution. Data from experiment 2W.

phate content was reduced even further. At this point the number of cells produced by a division is equal to the number that die or become dormant. But since death does not entail the immediate return of phosphate to solution, the available phosphate for uptake is only that which remains in a dissolved state. It was found experimentally that further divisions could take place even below 0.1 microgram atoms of phosphate-phosphorus per liter.

Figure 4 depicts typical relationships between the dissolved phosphate concentration and the phosphate fixed per diatom in a single experimental series. The phosphate content per diatom is observed to vary nearly linearly with the phosphate in the surrounding medium. We may thus write that

$$P_d = A + BP_s, \tag{3}$$

where A and B are constants. A would then be related to the minimal phosphate concentration per diatom, while B would be a function of the initial phosphate concentration for any given experiment. It should be noted that all points plotted in Figure 3 were taken while the diatoms were on the exponential growth curve.

To check the accuracy of the minimal phosphate content per diatom as determined radiochemically, diatoms were grown in the nutrient medium with no added phosphate. The diatoms were assayed daily for population density. At the

TABLE III

Culture flask	Final cell population, diatoms/ml.	P_d, micro-gram-atoms/diatom $\times 10^8$
1	25,000	4.3
2	22,000	6.6
3	28,000	5.1
4	28,000	3.1
5	25,000	4.9
6	25,000	5.7

approach of the leveling-off period, the diatoms were filtered onto a Whatman No. 42 7-centimeter filter paper disc. The total phosphate content of the diatoms contained in the known volume of the culture flask was determined by digesting the filter paper in 5 ml. of a 2:2:1 nitric: perchloric: sulfuric acid solution until fumes of sulfuric acid appeared. The residue was diluted to 100 ml. and an aliquot assayed for inorganic phosphate. Blank filter paper was simultaneously treated to determine the correction factor due to phosphate contained in the filter paper and the reagents. The corrected results of P_d obtained by this procedure are listed in Table III. It is manifest that they may be compared favorably with the minimal values obtained by the radiochemical method. The initial phosphate concentration was 1.1 microgram atoms of phosphate-phosphorus per liter, indicating that essentially all of the phosphate was utilized in diatom production.

One series of experiments was conducted on phosphate regeneration. The di-

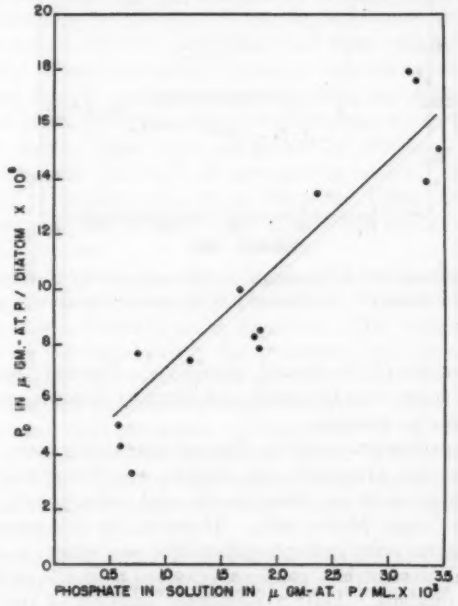

FIGURE 4. The phosphate fixed per diatom versus the phosphate concentration of the surrounding solution. Data from experimental run T.

atoms after reaching maximum growth were placed in a darkened box at 17° ± 2° C. with no stirring. Daily aliquots were taken and the supernatant liquid analyzed. As is shown in Figure 5, very little of the phosphate returned to solution after three weeks. This experiment was initiated at a low phosphate level. It would thus appear that the minimal phosphate is strongly bound. Although we may treat this experiment as somewhat of a laboratory phenomenon where the breakdown of the diatom is a function of the bacteria present, it nevertheless indi-

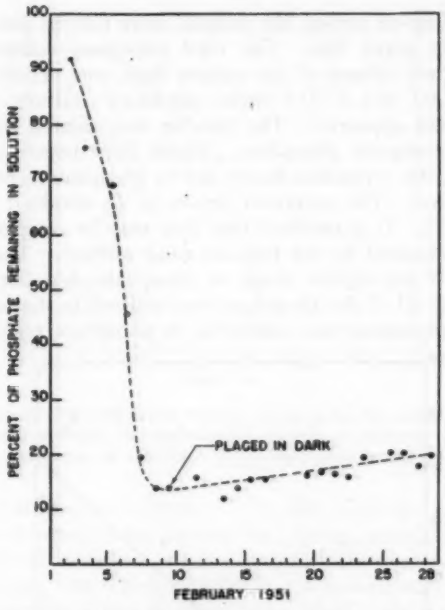

FIGURE 5. The regeneration of phosphate to the solution by diatoms deprived of light. The rapid uptake from February 2 to February 9 represents the diatom growth. Data from experiment 2Q.

cates the stable character of the bound phosphate. Further, in dynamic considerations considerably more time for death and decay of diatoms must be allowed for the return of phosphate to solution.

In some initial experiments involving diatoms containing more than the minimal amount of phosphate, the phosphate per diatom was determined by collecting a known number of organisms on filter paper, and subsequently assaying radiochemically under the Geiger-Muller tube. However, in this process, it was found that washing the diatoms with radiophosphate-free sea water, to free them of any adhering phosphate in the nutrient medium, removed as much as 50 per cent of the phosphate from the diatoms. Thus a significant fraction of the phosphate above the minimal amount appears to be loosely bound and readily exchangeable, and we may designate it as the labile phosphate.

Discussion

From this dynamic study of the assimilation of phosphate by the marine diatom *Asterionella japonica*, it is evident that these plants have a phosphate content dependent upon that of the environment. This phenomenon, not heretofore fully established, adequately explains the anomalies in phosphate content of diatoms in both cultural and natural populations. Lund's experiments on the fresh water species *Asterionella formosa* first suggested this relationship which he designated as phosphate storage. He found that cells grown in stock culture of 74 microgram-atoms of phosphate-phosphorus per liter contained 3.7×10^{-8} microgram atoms of phosphate-phosphorus per diatom, whereas the cells in stock cultures of only 19 microgram-atoms of phosphate-phosphorus per liter contained but 1.5×10^{-8} microgram-atoms of phosphate-phosphorus each.

By these radiochemical techniques the minimum phosphate content of a diatom can be ascertained, which in the case of the species studied was about 5×10^{-8} microgram-atoms of phosphate-phosphorus per cell. This phosphate may best be designated as "bound phosphate" or the phosphate which is not readily exchangeable with that of the surroundings. Such a condition as this must obtain whenever the phosphate is incorporated irreversibly into the protoplasm. The labile or readily exchangeable phosphate should in no sense be considered as storage since this phosphate level reflects the environmental phosphate through the uptake process.

Inasmuch as this single species was able to flourish in phosphate concentrations lower than the minimum levels found in the euphotic zone of the Pacific Ocean and in surface sea water to which no phosphate had been added, it appears that the inorganic phosphate content of marine waters is representative only of the total potential population that it could support, assuming no diffusion of this nutrient. From the data in Table II of the culture experiments it can be seen that there is a limiting growth factor other than phosphate or nitrate. Moreover, sea water media enriched a second time with all nutrients or with all nutrients but phosphate and nitrate failed to produce growth of diatoms. Using this same technique on other essential elements, the authors hope to establish further minimum mineral requirements of this diatom.

The relationship between phosphate uptake and the concentration of nitrate can be qualitatively seen from the experimental data. The initial nitrate concentration in all experiments was constant at an excess of 2000 microgram-atoms per liter. From Figure 4 we see that initially the phosphate per diatom is about 16×10^{-8} microgram-atoms phosphate-phosphorus per cell, where the phosphate in solution is 3.5 microgram-atoms phosphate-phosphorus/liter. From Table II, experiment 3T, we find that at a final phosphate concentration of 4.2 microgram-atoms phosphate-phosphorus/liter, the cell content is about 5×10^{-8} microgram-atoms of phosphate-phosphorus. Thus, a high nitrate/phosphate ratio is reflected in a high phosphate content of the diatom cells. This result is in agreement with the work of Ketchum (1939).

In the present study it was not considered essential or possible to have bacteria-free diatoms. First of all, only the exponential growth phase of diatom populations was studied. Accordingly, the biomass consisted almost entirely of actively growing plants. There was thus no substrate for bacterial growth. The remarkably close correspondence between the curves of phosphate depletion and

diatom growth would only result if the phosphate was entirely assimilated by the diatoms. The culture medium was autoclaved to remove any other competing living forms.

The diatom stock cultures which were used for inoculation were never freed of bacteria, inasmuch as they were healthy and normal cells. It seemed unlikely that any number of repeated washings could free these cells from bacteria which were attached between the frustule and the cell wall. Observations on decaying diatoms showed that the decay takes place within the frustule and the frustule does not open until the decay process is nearly complete. The standard procedure for determining sterility in diatom cultures is normally carried out on cells which have been repeatedly washed and never on aged cultures. It would seem that the only test for sterility should be one of direct observation as to whether the moribund cells decay.

Summary

1. Studies were made on the uptake of phosphorus by the marine diatom *Asterionella japonica*, utilizing radioactive phosphorus as a tracer.

2. The higher phosphate concentrations in the solution were linearly related to higher contents of phosphate in the diatom cells.

3. A minimal cell concentration of phosphate for further growth was established for this species.

4. Two types of phosphate were found in the cells: the minimal content which was strongly bound and not removed by water washing of the diatoms and a labile phosphate which could be removed. Experiments on the release of phosphate conducted on diatoms containing only minimal amounts of it, indicated that only a small percentage of the phosphate was returned to solution as soluble phosphate after three weeks.

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THE EFFECT OF MALEIC HYDRAZIDE ON THE EMBRYONIC AND LARVAL GROWTH OF THREE AMPHIBIANS

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Since Schoene and Hoffman (1949) first reported that maleic hydrazide (MH) inhibits plant growth, many investigators have extended and in general substantiated their findings. Greulach and Atchison (1950) reported that MH inhibits mitosis in onion root tips, Compton (1951) that it inhibits mitosis in pea seedlings, Girolami (1951) that it inhibits mitosis in flax, and Greulach (1952) that it inhibits mitosis in bean buds. Darlington and McLeish (1951) found that MH inhibited mitosis in the roots of several species of plants, and that it induced chromosome breakage in *Vicia faba,* but not in *Rhoeo discolor, Muscari palmosus,* or *Scilla sibirica.* The breakage was confined to the heterochromatin (instead of to the euchromatin as with X-rays), and no sticky chromosome surface was produced.

Since MH has a striking effect on cell division and growth of plants it would be of interest and value to learn whether MH has similar effects on animals. There is very little published information on the effect of MH on animals. Zukel (1951) reports that the oral LD 50 of MH to rats is 2.2 gm./kg. body weight, and that there is no irritation to the skin or mucous membranes of experimental animals from contact with MH. An earlier mimeographed release from the Naugatuck Chemical Company (producers of MH) stated that acute toxicity of MH to mice by intraperitoneal injection was 1.5 gm./kg. of body weight. Johnson, working at the University of North Carolina, has unpublished data which indicate that MH does not affect the growth of mice nor the growth of testicular tumors in mice. Young white mice sprayed daily for three weeks with six different MH concentrations ranging from 0.001 to 0.2 per cent, or given drinking water with the same concentrations of MH for three weeks, grew as rapidly as the controls and exhibited no toxic symptoms. Older white mice into which testicular tumors were transplanted were injected subcutaneously for ten days with 0.1 ml. of one of nine MH concentrations ranging from 0.05 per cent to the undiluted original solution containing 30 per cent MH by weight. No concentration of MH inhibited growth of the tumors, nor did any concentration produce symptoms of toxicity.

Experiment with Rana pipiens

Ovulation was induced in female frogs (*Rana pipiens*) by pituitary injections, and the eggs were fertilized by sperm from testes macerated in Ringer's solution. Two days later, when the embryos were in the neural tube stage, 15 embryos were placed in boiled and cooled tap water, 15 in a 0.1 per cent solution of MH, and 15 in a 0.05

[1] The authors wish to thank Dr. H. E. Lehman of the Department of Zoology, University of North Carolina, for suggestions concerning the experiments with Amblystoma.

per cent solution of MH. The MH was used in the form of the diethanolamine salt and was dissolved in boiled and cooled tap water, the same being true of the other experiments reported here. After 48 hours the embryos were transferred from the MH solutions to tap water and the embryological and larval development were observed over a period of six weeks.

Five days after the beginning of treatments the mean overall length was 10.5 mm. for the controls and both treated groups. The length of the body without the tail was 3.9 mm. for the controls, and 3.6 mm. for both treated groups. The body width was 3.2 mm. for the controls, 2.5 mm. for those treated with 0.05 per cent MH, and 2.4 mm. for those treated with 0.05 per cent MH, and 2.4 mm. for those treated with 0.1 per cent MH. These differences were not statistically significant. There was no observable effect of 0.05 per cent MH, but in the 0.1 per cent MH treatment there were retardation of eye development, smaller myotomes and gills, and a more sluggish response to light and touch than in the controls. Circulation as observed under a binocular dissecting microscope was apparently normal in both treated groups. The mortality rate in the 0.1 per cent MH was slightly higher than in the other two groups, but in the survivors the early minor differences in growth rates were overcome by the end of the experiment.

Experiment With Bufo sp.

Freshly laid eggs of an unidentified species of toad were collected in the field, and 75 embryos in the early blastula stages (with ectodermal epiboly incomplete) were selected and placed in 0.02 per cent MH, 75 similar embryos being placed in boiled tap water. After 48 hours the embryos in MH were transferred to tap water. Five days later four larvae from each group were selected for tail tip amputation and study of the rate and amount of tail tip regeneration, the remainder being retained for observations of growth and development.

The MH had no observable effect on embryonic or larval development, reflexes, or amount and rate of tail tip regeneration.

Experiments With Amblystoma punctatum

Embryos of the salamander, *Amblystoma punctatum*, were obtained in early cleavage to yolk plug stages. The jelly was removed and batches of embryos were placed in tap water which had been standing 48 hours, and in 0.5, 1.0, and 2.0 per cent solutions of MH. After 10 days all the gastrulae were transferred to standing tap water and their development was observed over a period of several weeks. In a second experiment the posterior third of the tail was amputated from a group of larvae 5 days after hatching. The larvae were divided into three equal groups and placed in tap water, 0.25 per cent MH, or 1.0 per cent MH. After 10 days the regenerated tail tips were clipped and then fixed and stained by the Costello-Henley (1949) method. The regenerated tissue was examined under oil for possible effects of the treatment on mitosis, and the number of mitotic figures per tail tip was determined, there being 8 control tail tips and an equal number in each of the two treated groups.

In the first experiment all the embryos in the 2.0 per cent MH died within 50 hours, but 35 per cent of those in the 1.0 per cent MH and 70 per cent of those

in the 0.5 per cent MH survived through the 10 day treatment period. Since only 84 per cent of the controls survived, the 0.5 per cent MH was apparently not particularly toxic. There was no superficial morphological difference between the controls and the surviving treated embryos, nor any significant difference in size. In the second experiment there was no significant difference in the size of the regenerated tail tips in the various groups. The mean number of mitotic figures per regenerated tail tip was 8.5 in the controls, 2.6 in the 0.25 per cent MH, and 3.0 in the 1.0 per cent MH. The differences between both treated groups and the controls were statistically significant at the 1 per cent level, but the two treated groups did not differ significantly from each other. There were many pyknotic nuclei in both treated groups, but none in the controls. All concentrations of MH used promptly killed the Daphnia and Cyclops introduced into the cultures for use as food by the salamander larvae.

Discussion

The results of these experiments indicate that MH does not have any significant or persistent effects on the growth and development of amphibian embryos and larvae similar to its effects on the growth of plants, even though several of the concentrations used on Amblystoma were much in excess of the concentration range of 0.02 per cent to 0.2 per cent usually used on plants.

In these experiments all concentrations of MH used were lethal to Daphnia and Cyclops, the 2.0 per cent MH was lethal to Amblystoma, and the 1.0 per cent MH was toxic to Amblystoma. The 0.1 per cent MH was somewhat toxic to Rana. Except for the latter concentration, all these concentrations were much in excess of those commonly used on plants. Taken in conjunction with the toxicity studies on rats and mice reviewed in the introduction to this paper, these data indicate that MH is toxic to animals only in rather high concentrations, considerably in excess of those used on plants. However, this does not take into consideration possible cumulative or delayed toxicity symptoms of lower concentrations. The sluggishness in some of the treated larvae of both Rana and Amblystoma could possibly be due to a decrease in the rate of respiration, since there is evidence that MH may decrease the rate of respiration in plants.

The data from the second experiment with Amblystoma would seem to indicate that MH did reduce the rate of mitosis in the regenerating tail tips. However, in the absence of any significant effects on total growth, in view of the fact that the two different MH concentrations had no different effect on the number of mitotic figures, and since starvation is known to induce pyknosis and inhibit mitosis in regenerating tail tips, the writers are of the opinion that the reduction of mitosis was not due to a direct effect of MH as in plants, but to starvation resulting from the lethal effect of MH on the crustaceans used by the Amblystoma larvae as food. Amblystoma larvae ingest only live, moving prey. It is planned to repeat this experiment with the addition of a starvation control group and with a wider range of MH concentrations, but in view of the basic complication involved it may be necessary to use other animals and techniques to clarify completely the effect of MH on mitosis in animals.

SUMMARY

1. A 2.0 per cent solution of maleic hydrazide was lethal to all *Amblystoma punctatum* larvae within 50 hours, but 35 per cent of the larvae survived 10 days in 1.0 per cent MH and 70 per cent in 0.5 per cent MH, while 84 per cent of the controls survived. All concentrations of MH from 0.25 per cent upward killed Daphnia and Cyclops introduced into the Amblystoma cultures as food. A 0.1 per cent solution of MH was slightly toxic to *Rana pipiens* larvae.

2. MH concentrations ranging from 0.02 per cent to 2.0 per cent had no significant effect on the growth of embryos and larvae of Rana, Bufo and Amblystoma. The full range of concentrations was not used on any one of the three species.

3. The number of mitotic figures in regenerating tail tips of Amblystoma was significantly less in those treated with either 0.25 per cent or 1.0 per cent MH than in the controls. Pyknosis was common in the treated tail tips but was absent in the controls. The writers are of the opinion that these are indirect, rather than direct effects of the MH treatment, and can be attributed to starvation following the death of the Cyclops and Daphnia provided as food for the Amblystoma larvae.

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THE METABOLIC RATES AND BODY TEMPERATURES OF BATS[1]

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Bats are among the hibernators—those mammals that pass the winter in a profound physiological state, characterized by: (1) reduction of the body temperature to virtually the ambient temperature, (2) reduction of the metabolic rate to an extremely low level, (3) reduction of other body processes (heart rate, respiratory rate, etc.) to minimal levels. These mammals are properly called heterotherms, for they are essentially poikilothermic when hibernating, but when awake and active maintain roughly constant body temperatures comparable to those of typical homoiotherms. It has long been known that bats reduce body temperature and metabolic rate whenever they become inactive (Hall, 1832). This reduction of metabolic rate would seem to be of great advantage to an animal that must expend such great amounts of energy as bats in flight must require. Pearson (1948) says: "Apparently because of small size and strenuous activities many bats have resorted to a metabolic parsimony of periodically lowered body temperature that allows their expensively gathered fuel to nourish them longer."

Despite the realization among naturalists that this remarkable condition exists in bats, there has never been any thorough study made to measure its dimensions, or to evaluate the possible impact on the lives of bats. The temperature coefficient of oxygen consumption has been studied in isolated tissues, in oxidative enzyme systems, in poikilothermic animals, and in newborn young mammals that have not yet developed the ability to regulate their body temperature. But it has not been possible previously to measure metabolic rate or oxygen consumption over a range of 40° C. in an intact, adult mammal. This paper presents the results of such measurements, made with the little brown bat (*Myotis l. lucifugus*) without subjecting the animals to thermal conditions outside of the range encountered in nature.

The investigation began by asking these questions: (1) To what extent do bats reduce their body temperature and metabolic rate during daily periods of inactivity? (2) Is there an essential physiological difference between this daily occurrence and hibernation? (3) Does this phenomenon separate bats from other hibernators on any physiological grounds? (4) What are the implications of this ability to reduce temperature and metabolism in relation to the life history of bats?

Question 4 will be answered in a later publication, as it is of greater interest in a mammalogical context than in a physiological one. However, the other three questions can be answered to a large degree here.

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I am indebted to many people for information and aid. Miss Mildred Wagner of this Center drew the figures. My wife, Ann, has read proof, checked references, and helped in innumerable other ways. Dr. P. F. Scholander gave counsel in the adaptation of his apparatus for this work. Dr. Laurence Irving, of the Article Health Research Center, has given encouragement and critical advice in the writing of this paper, and I am grateful to him for this and many discussions on similar matters.

To Dr. Donald R. Griffin, of Cornell University, I am most greatly indebted, for he first proposed that I do this research, and supervised, counseled, and criticized all phases of it. Furthermore, he has critically read both the thesis and this paper, and I have accepted nearly all of his suggested changes. However, all responsibility for statements made here is my own.

Materials and Methods

The bats used in this study were *Myotis lucifugus lucifugus* Kerr, the little brown bat. This species is found over much of North America, and is very common. Its habits are better known than those of any other American bat (Griffin, 1940; Wimsatt, 1945; Folk, 1940). It is found in hibernation in caves in northeastern United States during the winter months. It weighs from 5 to 9 or 10 grams.

Table I

Body temperatures of bats in relation to ambient temperature

Experimental temperature		Bat temperatures		Length of experiment	Rate of temperature change
Water bath	Air in vial	At start	At end		
°C.	°C.	°C.	°C.	min.	°C./min.
1.3	1.3	19.1	1.35	45	−0.39
23.0	23.1	24.7	23.2	30	−0.05
30.2	30.2	36.3	31.2	90	−0.05
37.0	35.5	35.7	37.5	15	+0.11

The bats used for these measurements were collected at caves in central Pennsylvania in winter, or in a large breeding colony near Ithaca, New York, in summer. Those bats taken in summer could be put into a torpid state even in July, merely by placing them in a refrigerator at 2-4° C. They were not fed during the time of their captivity, but were always kept in hibernation. High humidity was maintained in their quarters in order to minimize loss of water.

Apparatus. The determinations of oxygen consumption were made with a Scholander plastic semi-microrespirometer (Scholander, 1950). This had been modified for the present study by attaching the vials to the bottom so that the bats could hang in normal head downward position. Six bats could be measured at one time in the six separate units of this Lucite apparatus.

The body temperatures of some bats were measured with the bat in a similar-sized chamber. A small incision was made in the skin of the bat's abdomen, and a 30-gauge copper-constantan thermocouple inserted laterally along the body wall. This method of measurement of body temperature has been found to be quite close to the rectal temperature (Vernet and Metcalf, 1948). Reference junctions were in the air of the vial and in the water bath.

Procedure. The bats were removed from the cold box, weighed, and placed in the apparatus after an interval of time which was adjusted insofar as possible so that the bat would be placed into the glass vial with a temperature close to that at which the experiment was to be conducted. The experiments lasted for varying periods of time at the different temperatures, because when first placed in the chamber, the bat was active and consumed oxygen at a high rate. At 2° and 10° C., the steady state of oxygen consumption was not usually reached until eight hours had passed, while at 20° and higher it was reached much more quickly. At these higher temperatures death rates increased rapidly as the experiment lengthened, perhaps due to rapid depletion of already low nutritive reserves. Thus, the optimum length of the determinations at the various temperatures was selected at some point midway between the average time at which the steady state was reached and the average time of death.

The metabolic rate of interest in this study is obviously that of resting bats at various body temperatures. Hence the values obtained during the initial state of

TABLE II

Averages of oxygen consumption of Myotis l. lucifugus at various ambient temperatures

Ambient temperature	No. of bats	Average weight	Average length of steady state period	Minimum metabolism	Maximum metabolism	Average metabolism
° C.		*gms.*	*hrs.*	*ml. O_2/gm./hr.*	*ml. O_2/gm./hr.*	*ml. O_2/gm./hr.*
0.5	5	5.2	2	0.039	0.170	0.113
2.0	21	5.2	25.5	0.022	0.039	0.030
10.0	13	5.9	16	0.049	0.094	0.071
20.0	27	6.2	4.15	0.232	0.555	0.393
30.0	21	7.0	1.12	1.06	2.96	2.00
37.0	12	6.9	1.25	2.00	5.11	2.89
41.5	3	7.0	1.00	3.79	4.66	4.14
44.0	5	6.4	0.5	1.12	1.78	1.47

activity were excluded and the metabolic rates during the declining phase at the end of long experiments were also not considered to be relevant. All data presented below represent average values of oxygen consumption during steady state periods with no visible activity, periods within which the individual determinations of oxygen consumption show no extreme variation and no trend either upward or downward. In Table II is shown the average duration of this steady state period at various temperatures. As might be expected, much longer periods of constant metabolic rate could be obtained at lower temperatures.

EXPERIMENTAL RESULTS

The data collected in this study have been plotted in Figures 1 and 2. In addition, all similar data from the literature have been collected, re-calculated to the present units if necessary and plotted in order to find whether the present data are near to the normal condition. The data on metabolic rate taken from other workers are not shown here.

Body temperatures. A primary point to be established in each measurement was the body temperature of the bat. It would have been difficult to conduct all

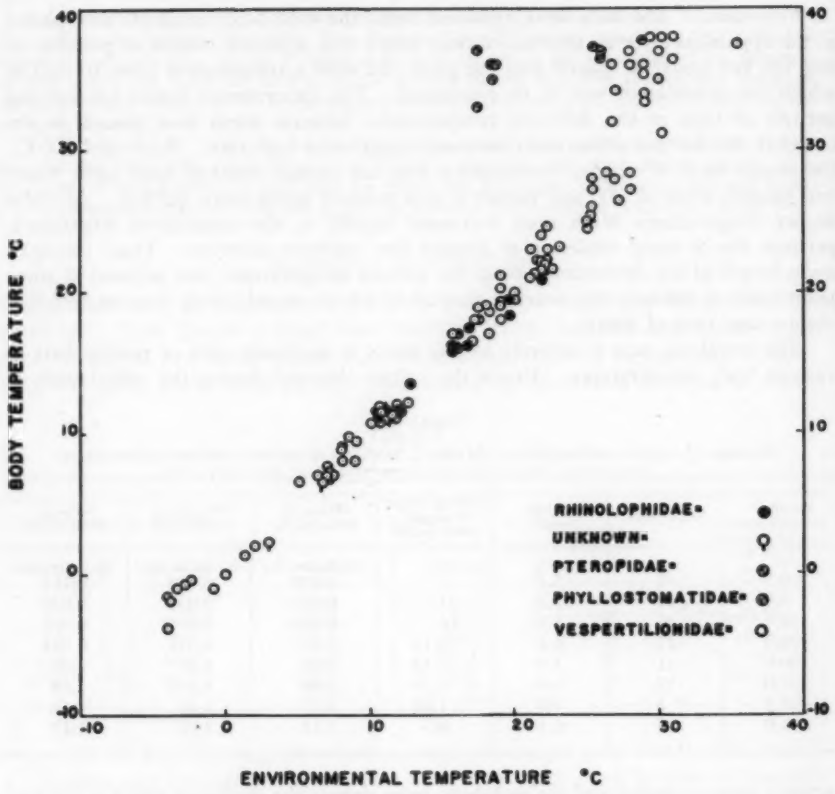

FIGURE 1. Body temperatures of bats in relation to environmental temperature. Data from the present study and all known literature. Four families, ten genera, and nineteen species are included. These are—Vespertilionidae—Hock, *Myotis lucifugus;* Reeder, 1949, *M. californicus;* Eisentraut (1933), *M. myotis, M. natteri, M. mystacinus, M. daubentonii, M. dasycneme, Nyctalus noctula, Eptesicus serotinus, Pipistrellus nathusii;* Eisentraut (1934), Delseaux (1887), and Kayser (1939), *Plecotus auritus;* Prunelle (1811), *Vespertilo murinus;* Burbank and Young (1934), *Nyctalus noctula;*—Rhinolophidae—Burbank and Young (1934), *Rhinolophus ferrum-equinum, R. hipposideros;* —*Pteropidae*—Burbank and Young (1934), *Pteropus geddei, P. giganteus, P. cotinus;*—Phyllostomatidae—Wislocki (1933), *Phyllostomus hastatus, Carrollia perspicillatum;*—Unknown—Pembrey and White (1896).

experiments with rectal thermocouples in the animals, particularly at the higher temperatures when the bats were alert and ready to actively struggle against any irritating attachment. Furthermore, a thermocouple attached to the body would undoubtedly have produced an increased metabolic rate in many of the animals. It was therefore decided to determine how closely the body temperature of bats approximated that of the water bath during the steady state period described above.

Accordingly, rectal temperatures were measured by copper-constantan thermocouples in bats at 4 water bath temperatures ranging from 1.3° to 37.0° C. The results are shown in Table I. It can readily be seen that by the end of the experimental period the rectal temperatures had come to a level closely approximating that of the water bath. The high initial rectal temperature at 1.3° C. bath temperature resulted from the fact that the bat was awake and warm when first placed into the apparatus; after 45 minutes, however, the rectal temperature had fallen to 1.35° C. and it seemed clear that the animal was in a condition comparable to the steady state period of the metabolic rate determinations.

In all cases there was a rapid approximation of the bat's temperature to the air temperature of the vial. This rapidity of change of body temperature increased as the difference between the original body temperature and the ambient temperature

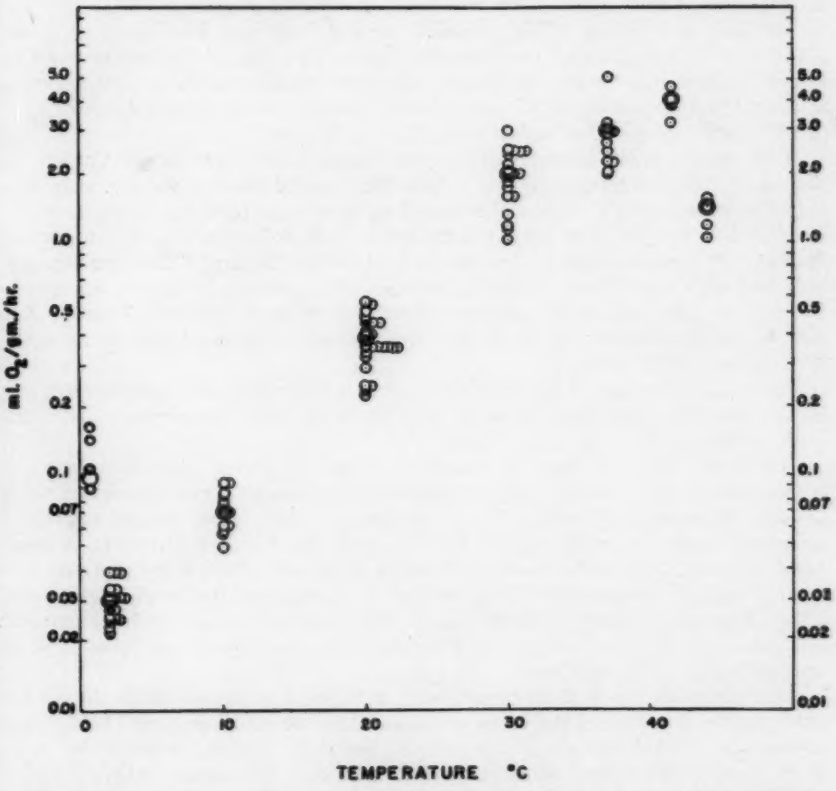

FIGURE 2. Semi-logarithmic plot of the metabolic rates of bats in relation to temperature. The temperature shown is environmental, but is very close to body temperature. Duplicate values are shown at the right of the line. Large circles are the average values.

increased. Table I shows these temperature determinations, including the rates of temperature change. It is apparent that body temperature closely approximates ambient temperature at all levels, but is closer at lower temperatures.

These results are in agreement with virtually all of the available information on body temperatures of bats. For example, Eisentraut (1934) found that the temperature of *resting* bats was nearly identical with the temperature of the surrounding air. Hence, during the steady state period of the present measurements, the body temperatures of the bats were, in all probability, less than 1 or 2° C. above the water bath temperature.

Figure 1 is a plot of all the body temperatures of bats that could be collected from this study and the literature, and that were not ruled out by the presence of activity at the time of the determination. It will be seen that there is an excellent approximation to a straight line. Furthermore, these data relate to several families and many genera of bats, and not only to a small closely related group.

Burbank and Young (1934) studied tropical fruit bats in Oxford zoos, and found that they maintained temperatures higher than that of the environment by means of shivering, flitting the wings, and other muscular activity. *Pteropus* sp. at room temperatures of 20° C. were always awake. At air temperatures of 17.5° to 18.5°, body temperature varied from 33.0° to 37.5° C.

Eisentraut (1938) studied temperature fluctuation in bats of the Cameroons. He found that the temperature at which they passed from a waking state to a lethargic state was *lower* than that found in temperate zone bats. However, he concluded that tropical bats behave in respect to daily reduction of body temperature just like the German bats he had studied. Further, he says, "Zusammenfassend lässt sich also sagen, dass die Beobachtungen an tropischen Fledermaüsen gezeigt haben, dass auch sie eine höchst unvollkommene Wärmeregulation besitzen, dass ihre Körpertemperatur und damit ihr Stoffwechsel weitgehend von Umgebungstemperaturen abhängig sind."

It thus appears that all bats exhibit a lack of thermoregulatory control when they become inactive, although they are capable of raising their temperature above that of the environment.

Metabolic rate. It may be expected from the above discussion on body temperature of bats that they will exhibit a variable metabolic rate dependent on the ambient temperatures to which they are subjected. Bats were exposed to ambient temperatures varying from 0.5° to 44.0° C., and their oxygen consumption measured. Figure 2 shows the results of these experiments. Each point represents the average oxygen consumption of one bat for the duration of the steady state period. Table II gives the average of all the steady state metabolic rates at each temperature, and gives also the average length of the period, the number of bats tested, and the average weight of the bats used.

The metabolic rate is at its minimum at 2° C., and is approximately double that value at 10° C. This is the range of temperature which Eisentraut (1934) characterizes as "deep hibernation." At 20° C., metabolic rate is five times that found at 10°, and by 30° there is another five-fold increase. Eisentraut (1934) delimited his "Tageschlaflethargie", or light hibernation as from 10° to 28° C. From 30° to 37° there is a smaller increase (1½ times) reflecting perhaps the fact that the bats are awake at the lower limit, although not yet with a "normal" body temperature. From 37° to 41.5° C., metabolic rate again increases 1½ times. This temperature

was selected for study because it has been stated to be the body temperature during flight (Burbank and Young, 1934). Furthermore, Barkow (1846) found body temperatures of bats when awake to be 41.0° to 41.6° C., although he did not say whether they were flying or not. At 44° C., the metabolic rate was lower than at 41.5°, and death occurred quickly. It thus appears that bats live on a narrow limit of temperature tolerance at the upper level, although there is a very great tolerance at the lower limits. However, the fact that cooling by evaporation could not be accomplished in the vials of the apparatus to the degree possible in the wild must be considered as a possible factor in this rapid death.

At 0.5° C., the metabolic rate quadrupled from that found at 2°. The bats thus increased metabolic rate as the temperature approached the freezing point, even though they were in deep hibernation. Lyman (1948) found that Syrian hamsters responded to temperatures of −1.5° to 1° C. in three ways; the majority increased oxygen consumption and cheek-pouch temperature, and finally awoke; two hamsters increased oxygen consumption to a steady rate 3–4 times that recorded before ambient temperature was reduced, and maintained a cheek-pouch temperature 1.7° to 3.4° C. above that of the environment for as long as 22 hours; four animals died in hibernation, perhaps due to poor condition of the animals. Note that these are the three possible reactions of hibernators to temperatures below 0° C.: to awake, to increase metabolic rate and body temperature without awaking, or to die.

Johnson (1929) subjected ground squirrels to temperatures near freezing and found that in many cases they awoke, and then returned to hibernation at these low temperatures. Some squirrels did not awake, however, but died when the temperature was at or below 0° C. Wyss (1932) found that dormice showed a great increase in metabolism below 0° C., while the body temperature was at least 2° above freezing. Dubois (1896) and Benedict and Lee (1938) reported conflicting results with marmots. Dubois states that they *always* awoke when subjected to near zero temperatures, while Benedict and Lee say that near zero temperatures *never* awakened their marmots.

Unfortunately, the experiment with bats at 0.5° was not continued long enough to show whether or not the bats would have awakened. One of the five bats tested did not increase its metabolism above the range of values found at 2°, while all others went much beyond this. It may be that these four would have awakened in time, while the one that did not increase metabolic rate would have died. It is interesting to note that Eisentraut (1934) has recorded body temperatures of bats as low as −3° C. Kalabuchov (1933) subjected bats to temperatures of −5° to −17° C., and reported that if freezing did not reach the vital organs (lungs, heart, etc.) the bats could be revived by thawing. It would be desirable to know the metabolic rates of bats under such conditions.

Discussion

Comparison with other data on bats. Metabolic rates of bats have been determined by several authors, either as oxygen consumption (Almeida *et al.*, 1926; Kayser, 1938, 1939, 1940; Morrison, 1948; Pearson, 1947), or carbon dioxide production (Delseaux, 1887; Koeninck, 1899; Pembrey and White, 1896), or both (Burbank and Young, 1934; Hari, 1909). These have been measured over a small range of temperatures with the exception of the series by Kayser (1940). The

range covered in his study is from 4° to 30° C. This is a study of *Nyctalus* (*Vesperugo*) *noctula*, and although the number of determinations at any one temperature is small, the study is comparable to the present series of measurements. (Kayser's series on *Pipistrellus* covers a range from 5° to 17° C., and contains more figures at each temperature. There is, however, such great variation that it cannot have involved resting bats in all cases. At 5°, for example, oxygen consumption varies as much as 150:1). I have not used Kayser's mean figures, because it seems that he excluded some data that are admissible from the point of view of my study.

When the data for *Nyctalus noctula* (Kayser, 1940) are compared with those for *Myotis lucifugus* as ml. 0_2/gm./hr., very close correspondence is found at the lower temperatures (2° and 4°, 10° and 12.5° C.). At 20° there is a fair correspondence between the two series, but at 30° the rate for *M. lucifugus* is four times that found for *N. noctula*. Two explanations seem possible: (1) the sample used by Kayser was too small, and does not represent the average metabolism; or (2) there is indeed a difference in the metabolic rate in the two species. The latter possibility seems to be the more likely, for my *minimum* metabolic rate was higher than Kayser's maximum (not included in calculations). Further, it is well known that the metabolism of animals (of the same general type, *e.g.*, mammals) varies inversely with increased size, whether plotted as weight or as surface area. *N. noctula* is three to four times larger than *M. lucifugus*, and calculations from the mouse-to-elephant curve of Benedict (1938) indicate that the difference in the metabolic rates of these two species at 30° is not far from the expected divergence for their weight variation. It may, however, be significant that no difference could be detected in metabolic rate per gram at lower temperatures.

Comparison with data on other hibernators. There are many studies of the temperature and metabolism of heterotherms during hibernation and when awake. Dubois (1896) found no fluctuation of the body temperature of marmots and other hibernators except in the fall, immediately preceding hibernation. Johnson (1928), on the other hand, states that ground squirrels (*Citellus* sp.) showed temperature fluctuation at all times of the year. When not hibernating the range of temperature was 32° to 41° C. The lower temperatures graded into those of slight torpor at about 29° to 32° C. Johnson (1931) considered that these fluctuations of body temperature in response to changes of ambient temperature were due (in part, at least) to the animal's "rather poorly adjusted heat regulating mechanism." Hamilton (1934) observed that the body temperatures of nine woodchucks varied from 34.9° to 40.0°, with the average 37.4° C. It thus appears that there is a lability of the body temperature of these heterotherms which extends over a small range, even when the mammal is not hibernating.

Kayser (1939) studied the effect of variations in ambient temperature upon the respiratory exchange of hibernators when they were awake. He used hedgehog, hamsters, marmot, spermophile, and dormice, as well as bats. All of these animals, with the exception of the bats, were capable of thermoregulation, although not to the same degree of precision as that found in the homoiotherms. Kayser also found that the basal metabolism of hibernators when awake was not distinctly different from that of the homoiothermic mammals.

Conclusions. It is now proper to sum up the evidence offered, in an attempt to answer the questions posed in the introduction. First, it is evident that bats can greatly reduce metabolic rate and body temperature. However, the normal daily

reduction has not been indicated thus far. The answer seems to be that these physiological measures of energy production are reduced to a level dictated by the ambient temperature. For a bat resting in 20° C., therefore, body temperature will be close to 20°, and metabolic rate will be near that indicated in Figure 2 for that temperature. This will be true at *any* ambient temperature, as long as the bat is in a resting state.

Question 2 asked whether there was a physiological difference between hibernation and the daily reduction of body temperature and metabolic rate. Hall (1832) called this latter state "diurnation," in contrast to "hibernation." Eisentraut (1934) called it "Tageschlaflethargie," or "leichter Lethargie," in contrast to "Winterschlaf" or "tief Lethargie," and considered that 10° C. was the dividing line between these two states. It does not seem to me that there is any essential distinction between them, and that those differences that do exist are those of degree, not of kind. To be sure, metabolic rate is greatly increased during exposure to temperatures above 10°, but this alone does not warrant erection of a new state, or the dignity of a new term. Rather it appears that the increased metabolism at these intermediate temperatures is a reflection of the increased function of tissues and organs of the bat, although it is still not capable of locomotion. In this respect, bats may be compared to the frogs, toads, and curarized dog of Krogh (1914), all of which showed an increase in metabolism at 20° although no movement occurred. To summarize, no basic physiological distinction can be shown to exist between the conditions found during the daily sleep of bats and those found during hibernation.

It seems apparent that the heterothermic mammals (other than bats) when not hibernating have physiological characteristics much like those of their non-hibernating relatives. Body temperature varies over a greater range, and metabolism reflects this, but both are in nearly all respects closely similar to the phenomena manifested by homoiotherms. Bats differ from other heterotherms in that their body temperature when at rest *at any season* always approximates that of their environment. The metabolic rate reflects this change, so that the metabolism is in direct proportion to the body temperature at all times. This condition is in opposition to that found in homoiotherms, where metabolism varies with external temperature, while body temperature remains nearly constant. Even other hibernators do not exhibit this phenomenon, for their metabolic rate does not vary in relation to body temperature when they are not hibernating. Rather, the metabolic rates during hibernation are of a quite different magnitude than those found during the normal state, and only these levels are clearly demonstrable, for when passing into or out of hibernation the metabolic rate at any one body or ambient temperature is a transitory affair (Lyman, 1948). Bats are thus the only mammals in which metabolic rate varies directly with body temperature. Question 3 must then be answered to the effect that bats are much different from other heterotherms in their lack of thermoregulation at all times of the year.

Summary

1. Oxygen consumption was measured in resting little brown bats, *Myotis l. lucifugus*, at ambient temperatures from 0.5° to 44.0° C. Body temperature was shown to approximate the ambient level under the conditions of the experiments, so

that in effect the metabolic rate was measured over very nearly this range of body temperatures.

2. The function relating oxygen consumption to body temperature is not linear; the Q_{10} is 2.94 from 2° to 10° C., 5.54 from 10° to 20° C., 5.09 from 20° to 30° C., 1.69 from 30° to 37° C., and 2.22 from 37° to 41.5° C. The ratio between highest and lowest resting metabolic rates is 138:1; the temperatures represented are 41.5° and 2° C.

3. Some notes on the reaction to temperatures very close to 0° C. are included. Bats increased metabolism over that observed at 2° C. when exposed to near-freezing temperatures. The experiment was not continued long enough to find whether or not they would eventually awake.

4. The data are compared with the findings of other investigators using several species of bats. There is a close correspondence between body temperature and ambient temperature in all species, except that the tropical forms increase heat production by muscular work. Bats of the temperate zone appear to show the usual effect of size on metabolic rate per unit weight at temperatures of 30° C. or above, as Benedict (1938) has demonstrated occurs in a wide variety of homoiotherms. At 2° and at 10° C. this difference is not apparent in a comparison between these measurements and those made by Kayser (1940) on a much larger bat.

5. It is concluded that there is no physiological basis for distinguishing the daily reduction of temperature and metabolic rate in bats from that found in hibernation, except in degree. It appears that this daily phenomenon is due to a lack of thermoregulatory control.

6. Bats are considered as distinct from other heterotherms in that, at all seasons of the year, their resting temperature and metabolic rate is dependent on the ambient temperature. This also is due to their lack of thermoregulation. They are the only mammals in which the resting metabolism is a direct function of the body temperature, rather than of the ambient temperature. Nevertheless, when active, they can maintain body temperatures of 40° C. or above.

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STUDIES ON CELL-DIFFERENTIATION: THE ROLE OF AUXIN IN ALGAE, WITH PARTICULAR REFERENCE TO RHIZOID-FORMATION IN BRYOPSIS[1]

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Auxin, the plant growth-hormone, has been shown to occur in species of brown algae, red algae, green algae and diatoms (van der Weij, 1933; duBuy and Olson, 1937; van Overbeek, 1940a, 1940b; Thimann *et al.*, 1942). Judging by these reports auxin is ubiquitous in the algae, yet relatively few experiments in the literature deal with the possible role of auxin in these plants; the overwhelming majority of auxin-experiments have used seed-plant material. Most of the auxin-experiments on algae have been concerned with cell multiplication in unicellular Chlorophyceae. Leonian and Lilly (1937) found inhibition with 1 p.p.m. of indole-acetic acid (IAA) and toxic effects with 10 p.p.m. on the growth of five different species in medium containing sugar. Yin (1937) reported significant increases in average cell *diameter* of *Chlorella vulgaris* in the presence of 1 to 40 p.p.m. IAA and sugar, although he found "no conclusive differences (in) the total growth of the cultures as determined by . . . dry weight determinations." A "positive stimulating effect" of IAA and other growth-substances was reported in a brief note by Brannon (1937) for *C. vulgaris, C. pyrenoidosa* and an *Oocystis* sp. grown in sugar-free medium; he claimed to have "some evidence that growth substances accelerated the rate of cell reproduction and increased the size of cells." Pratt (1938), testing a wide range of IAA concentrations, found no effect on the average cell diameter of *C. vulgaris*, although the cell number per mm.^{3} was increased up to 32 times in sugar-free medium. Brannon's full report (Brannon and Bartsch, 1939) confirmed Pratt's finding of an increase in cell-number in *C. vulgaris* when IAA was added, but now reported that it was "impossible to secure reliable data respecting the effect of a growth substance upon the size of (*Chlorella*) cells." They pointed out that the inhibition from IAA reported by Leonian and Lilly (1937) was probably due to the use of dextrose in the culture medium, since Brannon and Bartsch found fewer cells per mm.^{3} with dextrose plus IAA than they did with dextrose alone. Lilly and Leonian (1941) complicated matters once more by reporting no increase in total dry weight of *Chlorella* cultures grown in the presence of 10 p.p.m. IAA but in the absence of dextrose. They state that there was a "fairly dependable parallelism" between dry weight and cell number. However, Brannon and Sell (1945) reported *increases* in dry weight of more than 4 times from the addition of the same concentration of IAA. Their culture of *Chlorella pyrenoidosa* was reportedly a duplicate of the culture called *C. vulgaris* by Lilly and Leonian (1941). Brannon and Sell obtained "pronounced and persistent variations" in preliminary experiments with IAA, variations which

[1] This work was carried out during the summer of 1950 while a Lalor Fellow at Woods Hole.

disappeared when the IAA was recrystallized to a constant melting point of 165° C.; they suggest lack of purification of IAA as a possible basis for the discrepant results of Lilly and Leonian. Inhibitory or toxic effects of IAA on cell number were reported by Manos (1945) for *C. vulgaris*. Unlike most of the other workers, Manos makes no mention of either adjusting the pH or of buffering.

An extensive paper by Algeus (1946) confirmed much of the earlier work and cleared up most of the discrepancies. The great importance of pH in IAA experiments was first pointed out: *Scenedesmus obliquus* and a strain of *C. vulgaris* showed an increase in cell number upon the addition of IAA only at acid pH, the effect of IAA increasing with decreasing pH. Furthermore, there were consistent differences in response to IAA not only between species but also between different strains of the same species. Only three of the ten species tested showed increases in cell number from any IAA concentration within the 0.1 to 100 p.p.m. range. For each of these three species the concentration giving maximum response was different. The observation of Brannon and Bartsch (1939) that IAA increased cell number in sugar-free medium but not in medium containing sugar was confirmed, as was Yin's (1937) report that IAA increased cell *size*. However, Algeus found cell size increased in sugar-free medium only at concentrations of IAA (100 p.p.m.) which were so high that the normal increase in cell number was inhibited. And only one of the two tested species showed even this increase.

In summary, a few species of unicellular green algae have been found to show an increase in cell number at IAA concentrations of the same order of magnitude as are effective physiologically in seed-plants. A larger number of unicellular green algae showed no increase in cell number even at acid pH, without added sugar, and with a wide range of applied IAA concentrations.

Auxin experiments with algae which are morphologically more complex have been even fewer. Olson and duBuy (1937) cited experiments to show that the polarity of the germinating zygote of *Fucus* could be determined by a high local concentration of IAA. They found no increase in the number of rhizoids formed when IAA was added. Young *Bryopsis* plants were treated with IAA in sea water at various molar concentrations by Darsie (1939), but the only effect observed was inhibition of growth at the 10^{-3} *M* and 10^{-4}*M* concentrations. It should be mentioned, however, that throughout his work Darsie deliberately restricted his attention to growth and tropisms of the shoot only. Kylin (1942) found that IAA increased the number of cells formed by germinating zygotes of *Ulva* and zoospores of *Enteromorpha* at pH 8.2, a result that has been confirmed with respect to *Ulva* by Levring (1945). Davidson (1950) applied a range of concentrations of various growth substances to fronds of *Fucus* and *Ascophyllum* and to *Fucus* sporelings. He reported stimulation of terminal frond growth, except with indole-butyric acid on *Ascophyllum* fronds, and stimulation of general growth of *Fucus* sporelings with IAA, indolebutyric and naphthalene-acetic acids. However, auxins in lanolin usually inhibited frond growth and never stimulated it. Auxins had no stimulatory effect on intercalary growth of fronds of either genus, no matter what the concentration or method of application. The fact that the "inhibitor" iodoacetic acid stimulated *Fucus* sporelings in essentially the same pattern relative to concentration as did IAA suggests the advisability of controlling pH effects. This was not done.

Williams (1949) reported that IAA produced gains in wet weight of small discs cut from some portions of the blade in *Laminaria*. Here, too, the published

results are not unequivocal. There were no replications, no measures of variability; almost half of the IAA-treated samples disintegrated during the 16-day experimental period, and the largest wet-weight increase reported was only 18 per cent over the controls.

It appears, then, that while auxin is widely present in the algae, there is little evidence as to its role—if any—in algal morphogenesis.

It is reasonable to expect auxin to have somewhat similar roles in the various plant groups in which it occurs. Now, one of the most striking effects of auxin in flowering plants is the stimulation of root formation by high concentrations of auxin. The structure in algae which most closely corresponds to the root of a flowering plant is the rhizoid. Although the rhizoid is a mere protuberance from a single cell (instead of a multicellular organ with a very high degree of cellular differentiation and diversity like the root) yet the rhizoid is usually negatively phototropic, forms a close anchoring connection with the substratum, and is formed typically at the basal end of stem-like structures. These are characteristics which rhizoids share with roots. Hence, the following experiments were designed to determine if rhizoid-formation in algae is stimulated by auxin.

Materials and Methods

Bryopsis plumosa (Hudson) C. Agardh was used as experimental material. *Bryopsis* was chosen because its normal life cycle is well known, some aspects of its developmental physiology and morphology have been studied (Noll, 1888; Winkler, 1900; Steinecke, 1925; Darsie, 1939), it has already been shown to contain relatively large amounts of ether-extractable auxin (Darsie, 1939; van Overbeek,

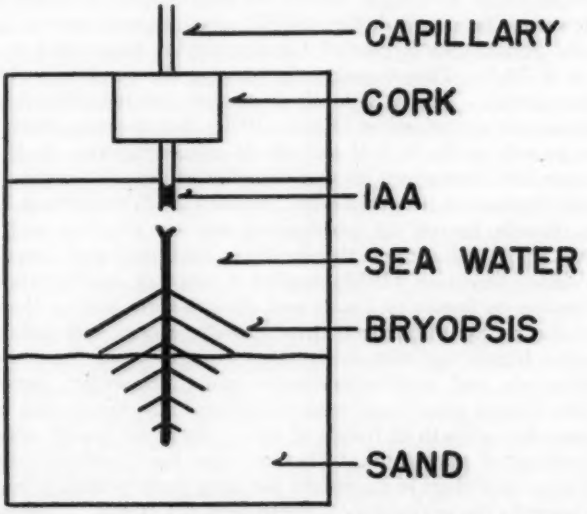

Figure 1. Diagram to show method of establishing a gradient of IAA along the long axis of *Bryopsis* plants.

1940a) and in its unicellular, multinucleate structure it seemed satisfactorily far removed from the organization of a flowering plant (Fritsch, 1935).

The general morphology of a young plant is shown diagrammatically in Figure 1. The vertically upright stem is positively phototropic, forms branches at the apex and negatively phototropic rhizoids at its base (Winkler, 1900; Steinecke, 1925). The chloroplasts are typically concentrated in the illuminated portions of the plant. The lumen of a young branch is in continuity with that of the main axis but by the time a branch is the 18th to 20th branch from the apex a cross-wall is beginning to form at its base. Thus, the branches furthest from the stem apex are morphologically separate cells.

Freshly collected, vigorous plants of *B. plumosa* were examined under the dissecting microscope and paired on the basis of close similarity in size, number of branches and rhizoids. One plant of each pair was placed in an inverted position in a shell-vial containing 10 ml. of sea water, as shown in Figure 1. The distal half of the branch-bearing portion was embedded in sand. A glass capillary was held so that the lower end was a few mm. above the base of the plant. The lower end of the capillary was filled with 0.7 to 1.0 mg. of IAA crystals. In replications of the experiment which used longer-stemmed *Bryopsis* plants, the lower opening of the capillary was held about 1 cm. above the oldest branches (*i.e.*, half-way down the unbranching portion of the main stem). The shell-vials shown in Figure 1 were placed in a water bath (at 21.5 ± 1° C.) to cut down convection currents which would disturb the diffusion gradient of IAA from the capillary tube. The relatively dim light from the windows was supplemented during the day with a 60 watt incandescent lamp held 18–24 inches above the shell-vials. The experiments lasted 12–15 days. Progressive dissolving of the IAA crystals occurred during the experimental period. There were usually some crystals left in the capillary even after 12 days.

To determine the extent to which the acid IAA changed the pH of the sea water, acetic acid was added to sea water to give the same molar concentration as was obtained with the highest concentration of IAA used (1.0 mg./10 ml. of sea water). (Acetic acid and IAA have the same pK.) The pH determinations were made with a Cambridge pH meter using a glass electrode. The untreated sea water had a pH of 8.01; addition of the acetic acid gave a pH of 7.25. Since all the IAA in the capillary was rarely dissolved by the end of each experiment and since the total possible drop in pH was so relatively small, pH effects were hereafter neglected.

At the end of the experiment the plants were first examined *in situ* with a horizontal microscope, then removed and compared under higher powers of the dissecting microscope.

Results and Discussion

When small, independently growing plants were prepared as described above, the results of all six replications showed that the superposition of an auxin gradient induces rhizoid-formation at the base of 77–95 per cent of the exposed (*i.e.*, aboveground) branches, while no rhizoids are formed at the base of branches in the control plants (Table I). Those branches which were below the level of the sand showed no basal rhizoids in either group of plants.

As *Bryopsis* plants continue their growth, secondary branches grow out on the primary branches and rhizoids usually develop at the base of the primary branches

near their region of connection with the main stem. Eighteen of these primary branches with their array of secondary branches were dissected off the original main stem, paired and then treated as were the individual plants above. Results are shown in Table II. The data indicate that the auxin-gradient still has a stimulatory effect on rhizoid-formation although the secondary branches are not nearly as reactive as are the young primary branches.

These experiments verify the hypothesis that the external application of auxin acts in *Bryopsis* to induce rhizoids at the region of relatively high local concen-

TABLE I

Effect of an external auxin-gradient on rhizoid formation in young Bryopsis plants. (The results of two of the six replications are shown.)

IAA-treated		Controls	
No. of exposed branches	No. of branches forming rhizoids	No. of exposed branches	No. of branches forming rhizoids
13	10	24	0
19	18	20	0

tration. The more extended hypothesis that auxin is the agent, naturally occurring in *Bryopsis*, which controls rhizoid-formation in the normal untreated plant is also supported by our experiments, particularly when they are considered in the light of reports of ether-extractable auxin in *Bryopsis* (Darsie, 1939; van Overbeek, 1940a) and of Darsie's finding that 73 per cent of the total plant auxin is in the basal (*i.e.*, rhizoid-forming) half of single-celled *Bryopsis* plants.

A possible obscuring factor was that despite the surface cleaning done under the dissecting microscope, the *Bryopsis* plants were, of course, not sterile; hence, the possibility must be recognized that the treatment with auxin may have acted indirectly on *Bryopsis* through the other organisms present. In my opinion this is unlikely.

The following interpretation of the development of *Bryopsis* can be advanced, based on these studies and those of previous researchers. *Bryopsis* is an organism

TABLE II

Effect of an external auxin-gradient on rhizoid formation by secondary branches of isolated primary branches

No. of pairs with more rhizoids on auxin-treated branches	No. of pairs with the same no. of rhizoids on each	No. of pairs with more rhizoids on the control branches
6	3	0

in which cytoplasm and chloroplasts can easily migrate from one portion of the coenocyte to another (Winkler, 1900; Steinecke, 1925). In the normal young plant auxin collects at the basal end of the coenocyte (Darsie, 1939) and there induces the formation of rhizoids. Once the older primary branches become morphologically separated from the main stem by the formation of thick cross-walls at their proximal end, the auxin produced in these branches can much less readily, if at all, move into the main stem. When the concentration of auxin becomes high enough at the proximal end of these separate branches rhizoids are formed there. Proximal rhizoids were never found in freshly collected material except when the "isolating" cross-wall was already present.

When *Bryopsis* is shaded (Winkler, 1900) the branch-tips continue their elongation as rhizoids instead of as "shoot" structures. Winkler (1900) and Steinecke (1925) have reported experiments which they believe show that this transformation is preceded by a migration of chloroplasts and cytoplasm out of the branch-tip and their replacement by cytoplasm which was in the rhizoidal end of the coenocyte. The experiments with auxin suggests that hormonal redistribution accompanies or is a consequence of the redistribution of cytoplasm.

Hence, it is suggested that one of the key differences between *Bryopsis* and seed-plants, the difference which is reflected in the strict developmental polarity of seed-plants and the labile polarity of *Bryopsis*, is that seed-plants have a strongly polar system for auxin-transport (Went and Thimann, 1937; Jacobs, 1950) while *Bryopsis* does not.

Summary

1. Application of indole-acetic acid, a naturally occurring auxin, to young *Bryopsis* plants results in the differentiation of rhizoids at the proximal end of most of the exposed "branches." Control plants formed no basal rhizoids.

2. Similar results were obtained with respect to rhizoid formation on secondary "branches" when the primary branches were excised from the original main stem and exposed to auxin. The response was not as strong under these circumstances.

3. Considered with the fact that auxin is present in large amounts in *Bryopsis*, with most of the auxin being in the lower (*i.e.*, rhizoid-forming) half of the plant, these experiments verify the hypothesis that the formation and distribution of auxin control rhizoid-formation in *Bryopsis* in a closely analogous way to auxin's effect on root-initiation in seed-plants.

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OBSERVATIONS ON THE MORPHOLOGY AND LIFE-HISTORY OF MICROPHALLUS LIMULI N. SP. (TREMATODA: MICROPHALLIDAE)

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During the summer of 1950, Dr. T. H. Waterman, while studying the finer structure of the eyes of the horseshoe crab, *Limulus polyphemus,* observed small cysts, each of which contained a larval worm. Cysts containing living larvae were referred to me for study and possible identification. The wall of the cyst is very tough and it was impossible by mechanical means to liberate the larvae without injury. Some of the cysts were left in sea water and two days later two of them had ruptured, freeing the larvae which were alive and active. The worms were almost sexually mature; the testes and seminal vesicles contained motile spermatozoa. The excretory system was traced completely and the morphology of the metacercariae showed them to be a species of *Microphallus.* An abstract of the findings was presented at the summer meeting of the Marine Biological Laboratory (Stunkard, 1950).

Twenty-eight specimens of *L. polyphemus,* collected in Pleasant Bay, Orleans, Mass., and with a size-range from 5 cm. carapace length to fully grown individuals, were examined and all were infected. Cysts were present in the connective tissue of the digestive gland and other organs as well as in the eyes, and in all of them the larvae were apparently representatives of a single species.

Cysts were fed to baby chicks, half-grown white mice and young golden hamsters. One chick was examined 2 days and another 5 days after feeding, but no worms were found. Examination of the birds' feces did not yield eggs of the parasite and birds killed later than 5 days after feeding were free of infection. One mouse died 12–16 hours after the first feeding and many excysted metacercariae were found in the initial portion of the small intestine. Another mouse, killed 2 days after feeding, contained worms with eggs in their uteri and all of 6 mice examined 2–4 days after feeding contained mature worms. After 5 days the number of worms diminished and none were found after 9 days. No worms were found after subsequent feedings of metacercariae, indicating a possible immunity from the first infection. The feeding of cysts to golden hamsters gave results substantially like those with mice. From these feeding experiments and the rapid elimination of the worms, it appears that mice and hamsters are not natural or normal hosts of the parasite. However, the recovery of sexually mature specimens makes it possible to describe the species and compare it with other related ones. The specimens can not be assigned with certainty to any known species and accordingly they are described as a new species for which the name *Microphallus limuli* is proposed. Specimens must be designated in some way and since it is simpler and easier to drop a name in syonymy than to distinguish between different species which have

been erroneously included under a single name, no serious inconvenience will result if the present specimens should eventually prove to belong to a previously described species.

Description

Cysts (Fig. 1) removed from *L. polyphemus* were oval, colorless, 0.16–0.2 mm. in length and 0.12–0.18 mm. in width. The wall was 0.009–0.011 mm. in thickness, hyaline, and resolvable into two layers, approximately equal in thickness. This wall was probably produced by material from cystogenous cells of the cercaria. It was enclosed in a thin, colorless, fibrous coat, probably deposited as a reaction product by the host.

Released from the cyst a metacercaria, under moderate coverglass pressure, measured 0.24–0.40 mm. in length and 0.10–0.20 mm. in width. Fixed, stained

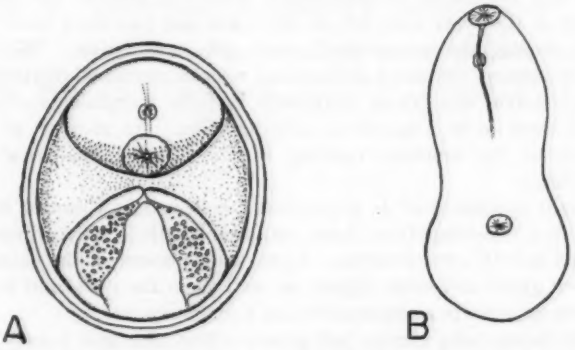

Figure 1. A; metacercaria in cyst, from *L. polyphemus*. B; outline drawing of fixed and stained specimen, 0.314 mm. long, with 84 eggs in the uterus, to show characteristic shape when elongate.

and mounted, this larva measured 0.30 by 0.19 mm. In it the testes, ovary and vitellaria had attained almost complete development; the testes and seminal vesicle contained motile spermatozoa. The size and location of the organs are shown in Figure 2. The flame cell formula is $2[(2+2)+(2+2)]$, which so far as known is characteristic of all members of the family Microphallidae. The cuticula bears imbricate, flattened spines which become progressively smaller posteriorly and may be absent behind the acetabulum. In living specimens the edges of the body tend to bend ventrally, forming a rim around an elongate depression. Typically there is a slight constriction at the level of the bifurcation of the digestive tract, and the body may become pyriform with either the anterior or posterior portion wider than the other. In front of the digestive ceca the body is filled with minute, unicellular glands.

Sexually mature worms (Figs. 1B; 4) from mice and hamsters are very little, if any, larger than the metacercariae. The suckers and individual organs are no larger in adult worms than in the larger metacercariae. The only increase in size

results from a bulbous expansion of the posterior one-third to one-half of the body, which may be almost spherical and when bent ventrally produces a claviform appearance. The increase in size of the posterior region is caused by the development of the uterus and the accumulation of eggs in it. More than 100 eggs have been counted in a single worm. The increase in thickness and massing of eggs obscure the organs in the posterior portion of the body and they are more conspicuous in the metacercaria.

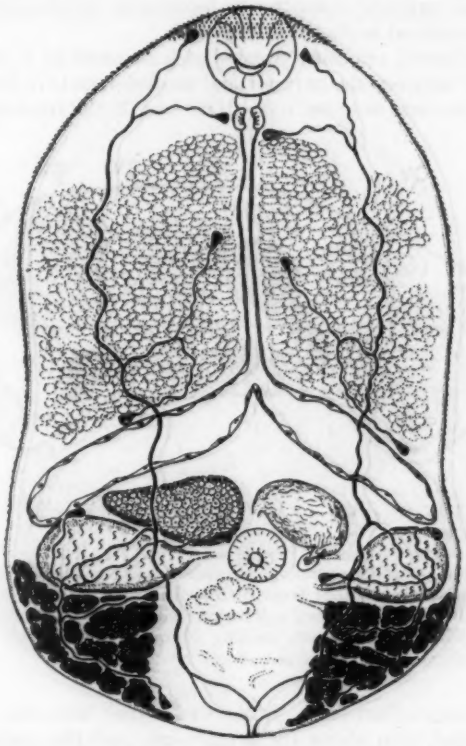

FIGURE 2. Fixed and stained metacercaria, 0.32 mm. long, ventral view, flattened to show excretory and genital structures.

Adult specimens, when alive and under moderate coverglass pressure, measure 0.26–0.40 mm. in length and 0.15–0.20 mm. in greatest width. The terminal egg-filled portion of the body is always wider and thicker than the flatter anterior region; otherwise the shape is unchanged from that in the metacercaria. The acetabulum measures 0.029–0.035 mm. in diameter and is located about one-third of the body length from the posterior end, a little farther forward relatively than in the metacercaria. The mouth is subterminal; the oral sucker is 0.035–0.045 mm. in diameter; the prepharynx is 0.01–0.033 mm. in length; the pharynx is 0.017–0.021 mm. in

diameter; the esophagus is 0.1–0.13 mm. in length; the intestinal crura, which are preacetabular, diverge at a wide angle and measure 0.08–0.10 mm. in length. Fixed and stained specimens are somewhat smaller; the smallest gravid worm is 0.26 mm. long; 0.11 mm. in greatest width; in diameter the oral sucker is 0.025 mm., the acetabulum is 0.0225 mm., and the pharynx is 0.012 mm. The uterus of this worm contains 14 eggs.

The excretory system is unchanged from that in the metacercaria. The pore is terminal; there is a preporal sphincter, a bicornuate bladder, from which the collecting ducts pass anteriad as shown in Figure 2.

The testes are lateral, opposite, situated at least partially in the acetabular zone and about midway between the anterior and posterior ends of the enlarged portion of the body; they are oval to ovate, 0.05–0.065 mm. in the transverse axis and 0.03–

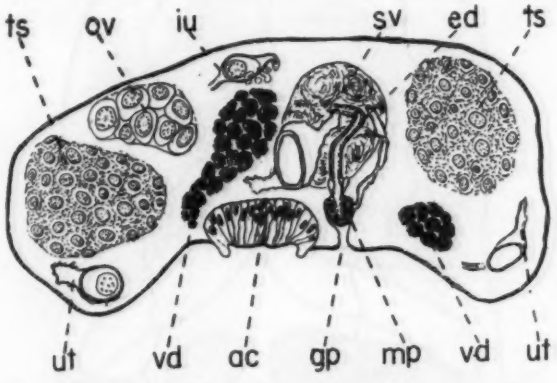

FIGURE 3. Composite drawing of two adjacent, slightly oblique, 10 μ sections, to show relations of acetabulum, gonads, genital pore, male papilla, vitelline ducts (one of which is enlarging before joining with the one from the opposite side), and dorsal to it the initial coil of the uterus containing a newly formed egg; ac, acetabulum; ed, ejaculatory duct; gp, genital pore; iu, initial coil of uterus; mp, male papilla; ov, ovary; sv, seminal vesicle; ts, testis; ut, uterus; vd, vitelline duct. Parenchyma not shown.

0.04 mm. in the longitudinal one. Sperm ducts arise from the median faces, pass mediad and anteriad, join above the acetabulum, and the common duct expands into a large, transverse, oval seminal vesicle which may be as large as one of the testes and is located medially or slightly to the left in the area directly behind the digestive ceca. From the vesicle, an ejaculatory duct curves posteriad, mediad, and ventrad. It terminates in the male papilla which opens into the genital atrium near the left margin of the acetabulum (Fig. 3). The distal portion of the seminal vesicle and the ejaculatory duct are enclosed in glandular cells. The male papilla is very small, 0.006–0.0075 mm. in diameter and 0.012–0.014 mm. long.

The ovary is oval to triangular, longer transversely, 0.06–0.065 by 0.036–0.045 mm., situated in the area bounded by the right cecum, the right testis, the acetabulum and the seminal vesicle. The details of the female reproductive ducts were observed in serial sections. The oviduct arises from the postero-median face of the ovary and

coils posteriad and ventrad; there is a small enlargement which contains spermatozoa and from which Laurer's canal winds dorsad to open near the midline, just behind the level of the acetabulum. Shortly after the origin of Laurer's canal, the oviduct turns dorsad and anteriad, receives a short common vitelline duct, and expands into the ootype. Mehlis' gland consists of only a few cells. The vitellaria are large, lobed glands, situated on either side, filling the regions between the testes and the posterior wall of the body. Ducts from the two sides pass mediad and anteriad; they unite to form the common vitelline duct which opens into the oviduct as noted

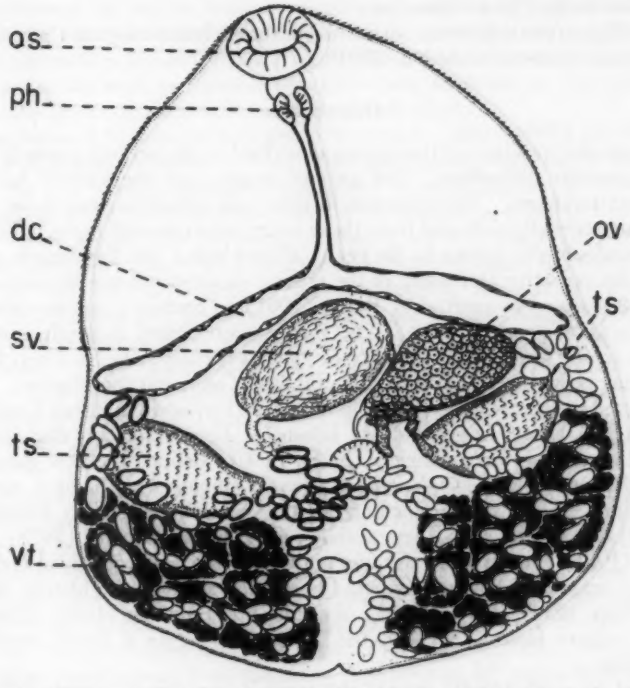

FIGURE 4. Fixed and stained specimen, 0.295 mm. long, with 120 eggs in the uterus, dorsal view, flattened to show details of the reproductive organs; dc, digestive cecum; os, oral sucker; ov, ovary; ph, pharynx; sv, seminal vesicle; ts, testis; vt, vitellaria.

above. The initial portion of the uterus is dorsal to the acetabulum (Fig. 3). The uterus passes posteriad in a sinuous course near the median plane almost to the end of the body, then coils anteriad, ventrally, along the right side of the body to the level of the cecum, turns posteriad and passes backward mediad to the ascending limb; near the posterior end of the body the uterus crosses to the left side of the body where it forms a loop similar to the one on the right side and the recurrent limb turns mediad to open into the genital atrium. The course of the uterus becomes more coiled and irregular as it is filled with eggs. The eggs are oval to ovate,

operculate, 0.016–0.02 by 0.009–0.011 mm., and some of them have a very small knob at the antopercular end. Eggs in the initial portion of the uterus are almost colorless; those on the left side of the body are bright yellow. Mature eggs contain ciliated miracidia. In the metacercaria, the vitelline follicles form a compact triangular mass, extending on each side from the testis to the posterior end of the body; in gravid specimens the vitellaria and testes are enclosed by coils of the uterus, which in part obscure their outlines.

Second intermediate host: *Limulus polyphemus.*

Definitive hosts: experimentally, white mice and golden hamsters.

First intermediate host: unknown.

Type and paratype specimens: deposited in the Helminthological Collection of the U. S. National Museum, slide No. 47592.

Discussion

The taxonomic position of the species described in the present paper is obscured by certain peculiar difficulties. The asexual stages, and the natural hosts of the adult are yet unknown. Development in mice and hamsters may have produced specimens substantially different from those maturing in normal hosts. The present specimens undoubtedly belong to the genus *Microphallus,* but the generic concept is still somewhat tentative and many of the specific descriptions are incomplete. The genus *Microphallus* was erected by Ward (1901) to contain a species described by him (1894) as *Distoma opacum.* The worms were found in fresh-water fishes: *Amia calva, Ictalurus punctatus,* and *Perca flavescens* from Lake St. Clair, Michigan. Metacercarial stages were encysted in crayfish, *Cambarus propinquus.* Stossich (1899) erected the genus *Levinsenia* and included *D. opacum* in it, but Looss (1899) argued that *D. opacum* Ward did not belong in *Levinsenia* and that probably it represented a genus as yet unnamed. Ward (1901) defined the new genus *Microphallus* and proposed a new subfamily, Microphallinae, to contain it and *Levinseniella.* The subfamily Microphallinae was included in the family Heterophyidae Odhner, 1914 by several subsequent authors, including Fuhrmann (1928), although Travassos (1921) had removed the subfamily from the Heterophyidae and elevated it to family status. The more recent morphological and life-history studies of Rankin (1939a, 1939b, 1940a, 1940b), of Cable and Hunninen (1940), Baer (1943) and other authors have established the Microphallidae as a family distinct from Heterophyidae.

As noted, Stossich (1899) erected the genus *Levinsenia* to contain four species: *Distomum brachysomum* Creplin, 1837; *D. macrophallus* v. Linstow, 1875; *D. pygmaeum* Levinsen, 1881; and *D. opacum* Ward, 1894. In the same year, Lühe and Looss, independently, designated *L. brachysoma* as type of *Levinsenia.* The next year, Jägerskiöld (1900) proposed *L. pygmaeum* as type of the genus. Ward (1901) designated *D. opacum* as type of a new genus *Microphallus;* he stated that *Levinsenia* Stossich was preoccupied (*Levinsenia* Mesnil, 1897) and that Stiles and Hassall were to propose the name *Levinseniella* to replace it. Although the paper by Stiles and Hassall did not appear until 1902, the name *Levinseniella* was validated by Ward (1901) with *Levinseniella brachysoma* as type species. Jägerskiöld (1901), although aware of the announcement by Ward, proposed the name *Spelotrema* for the invalid name *Levinsenia,* with *S. pygmaeum* as type. He stated, p.

982, "Ich sehe aber aus Ward's Aufsatz: On the Structure of the Copulatory Organs of *Microphallus* n. g. (Studies from the Zoological Labor. The Univ. of Nebraska), dass Stiles mir zuvorgekommen ist, und lasse daher, obgleich ich den Aufsatz von Stiles noch nicht habe finden können, seinen Namen *Levinseniella* gelten." Jägerskiöld (1904) listed *Levinseniella* as a synonym of *Spelotrema* although he (1907) recognized the two as distinct genera. Jägerskiöld (1901) proposed *Spelotrema* as a replacement for *Levinsenia* and regarded the two as identical, but actually this is not the case since *L. brachysoma* was designated as type of *Levinseniella* and *L. pygmaeum* as type of *Spelotrema*, and the two species are not congeneric. The name *Spelotrema* has had an unsavory reputation and the history of the name and of the generic concept were reviewed by Cable and Hunninen (1940). They discussed the nomenclatorial problem outlined above. However, the distinctness of the species allocated to *Spelotrema* and those included in *Levinseniella* was demonstrated by the studies of Rankin previously cited.

The problem of *Spelotrema* appears to be solved. Baer (1943) stated that there are no morphological differences which distinguish *Spelotrema* from *Microphallus*, and that the two genera had been maintained because species of *Spelotrema* are typically parasites of birds whereas those of *Microphallus* occur in fishes and amphibians. Baer argued that difference in host is not a proper criterion for generic distinction, since the metacercariae of these species are encysted in crustaceans which are eaten by both birds and fishes. The metacercariae are well developed and become sexually mature in a few days after ingestion by the definitive hosts. Moreover, certain species mature in mammals as well as birds and host-specificity is not at all precise. Since *Microphallus* antedates *Spelotrema*, the latter name was suppressed as a synonym and the species formerly included in *Spelotrema* were transferred to *Microphallus*. The identity of *Microphallus* and *Spelotrema* has been accepted by Rausch (1947), Rausch and Locker (1951) and by Cable and Kuns (1951).

The number of valid species in the genus *Microphallus* is yet uncertain. Osborn (1919) described *M. ovatus* from *Micropterus dolomieu* taken in Lake Chautauqua, New York. Strandine (1943) compared specimens of *Microphallus* from *Amia calva* and *Micropterus dolomieu* of Lake Lelanau, Michigan, and found no significant difference between *M. opacus* and *M. ovatus*. He reported overlapping variations in total size, size of acetabulum, extent of spination, length of ceca, and size of eggs in the two previously accepted species. Rausch (1946a, 1946b) discussed morphological variation and host-specificity of species in the genus *Microphallus* and reported natural infections by *M. ovatus* in three species of turtles and in the racoon. He (1947) found that microphallid cysts from crayfish fell into two distinct size groups, the larger of which (1.3 by 0.9 mm.) agrees with the measurements given by Ward (1894) for cysts of *M. opacus*. Although the cysts were of two sizes, even from the same crayfish, the metacercariae were reported to be morphologically identical and all were able to mature when fed to suitable experimental hosts. He was unable to excyst the larvae by mechanical means, but found that they emerged in 48 hours when placed in Ringer's solution with fragments of the gland in which they were found. On release, they began to copulate and when left in normal saline, Ringer's solution, or media devised for culture of protozoa, they matured and produced large numbers of apparently normal eggs. He fed metacercariae to various kinds of possible hosts; the cysts failed to hatch in chicks

and toads; the metacercariae emerged but were voided in an immature condition by certain snakes. Sexually mature worms were recovered from two species of turtles, two snakes, the opossum and the racoon, but only negative results were obtained with salamanders, frogs, chicks, albino rats and a skunk. Adults from the two types of cysts differed in size, but since they were otherwise so similar, Rausch concluded that they belong to a single species. Furthermore, he suggested that *M. gracilis* Baer, 1943, may also be identical with *M. opacus*. These experiments of Rausch are of fundamental significance, since they exemplify the surest method of determining specific relations among parasitic worms. His data suggest that there are actually two species represented in his material, and *M. ovatus* may be distinct from *M. opacus*.

Diagnoses of previously described species in the genus *Microphallus* (syn. *Spelotrema*) were made by Rankin (1940a), Cable and Hunninen (1940), Baer (1943) and Rausch and Locker (1951). Earlier authors, Odhner (1905), Nicoll (1907), and more recent ones, Rankin, Cable and Hunninen, and Baer, regarded the size of the male papilla as a diagnostic, specific feature, whereas Rausch and Locker emphasized the length of the digestive ceca and the relative size of suckers. Rankin (1940a) described the life-cycle of *Spelotrema papillorobusta* n. sp., and listed the specific details of five previously described species. Cable and Hunninen (1940) tabulated the specific features of five species which they compared with *Spelotrema nicolli*, a species described by them as new and for which they traced the life-history. Baer (1943) described *M. gracilis* n. sp., from *Neomys fodiens*, suppressed *Spelotrema* as a synonym of *Microphallus*, and recognized fourteen species, thirteen of which were distinguished in a key. The fourteenth species, *M. minus* Ochi, 1928, was not included as the description was published in Japanese. The genus *Monocaecum* Stafford, 1903, was suppressed as a synonym of *Microphallus* and its only species, *M. baryurus* was listed as possibly identical with *M. ovatus*. Baer noted that the metacercariae attain almost complete maturity in their crustacean intermediate hosts, that they become gravid in a few hours in the anterior portion of the intestine of various cold-blooded and warm-blooded hosts, and that they diminish in numbers from day to day as they migrate toward the posterior portion of the intestine. To account for these facts, he suggested that the metacercariae have developed a "progenèse retardée" or "néoténie naissante," and that the cold-blooded hosts may be "hôtes d'attente" to which the parasites have become adapted. Rausch and Locker (1951) described *M. enhydrae* n. sp., from the sea-otter, *Enhydra lutris*, and recognized fourteen species in the genus *Microphallus*.

The present specimens agree most closely with the description of *M. claviformis* (Brandes, 1888), described originally from the rectum of *Tringa alpina* at Halle, Germany. Brandes stated that the body was often constricted into two regions, with a flattened anterior portion about twice as long as a swollen posterior portion, which gave the worms a club-shape and suggested the specific name, *Distomum claviforme*. The description and figure give an incomplete, inadequate, and probably erroneous characterization of the species. Lühe (1899) stated, "Bei *Dist. claviforme* Brds., welches sich durch die auffällige Länge von Praepharynx und Oesophagus auszeichnet, ist die Lage und Form der Dotterstöcke und die Lage des Genitalporus vollkommen unbekannt, so dass die Art meines Erachtens zur Zeit in ein System nicht eingereiht werden kann." Jägerskiöld (1900) added, "Was aber *Distomum claviforme* Brandes betrifft, so bin ich, mit der Ansicht Lühe's anschliessend, eher

geneigt zu glauben, dass es zu den nie zu identifizierenden Arten gehört, wenn es nicht etwa dem Autor selbst gelingen wird, es wiederzufinden." Jägerskiöld suspected, and justifiably, that Brandes had misidentified the internal organization, that the reported anterior testis was the seminal vesicle, that the posterior testis was the cirrus sac or gonotyl, and that the actual testes and vitellaria were obscured by the windings of the uterus. Brandes suggested that the worms might be the adult stage of a metacercaria encysted in the crab, *Carcinus maenas*.

What was presumed to be the same species was redescribed by Nicoll (1907) as *Spelotrema claviforme* from *Pelidna* (*Tringa*) *alpina* and *Aegialis histicula*. He noted that the sexually mature worms were smaller than the metacercariae encysted in *C. maenas*, thus nullifying the suggestion of Brandes. In a second paper, Nicoll (1909) restated the specific diagnosis, included a figure, and added *Anthus obscurus*, *Numenius arquata*, *Moticilla flava* and *Larus ridibundus* as new hosts of the parasite. Apparently Brandes and Nicoll are the only authors who have published original observations of the species, although it had been transferred to *Brachycoelium* by Stossich (1892) and to *Lecithodendrium* by Stossich (1899).

The only morphological feature in which the present specimens differ from the description of Nicoll is in the size of the male papilla, which is only about one-half the size of that in the specimens described by Nicoll. The worms from *Limulus* may indeed be specifically identical with those described by Nicoll, and at first they were referred to *M. claviformis*. But bionomic features seem to preclude the allocation of the specimens to that species. In all other microphallids for which the life-history is known, the metacercariae occur in crustaceans. While larvae of this species may encyst in crustaceans, the intense infection in *Limulus* suggests that the horseshoe crab is the natural host, and *Limulus polyphemus*, a living representative of the Xiphisurida, is a chelicerate form related more closely to the pycnogonids and the scorpions than to the crustaceans. It is far removed, zoologically and phylogenetically, from the Crustacea. Moreover, *Limulus* does not occur on the coast of Europe and some other animal must serve there as the second intermediate host of *M. claviformis*. Adult stages of the present species have so far been recovered only from mammals; and possible bird hosts, other than the chick, have not been tested. If the species is actually *M. claviformis*, it should mature in *Larus ridibundus* when young birds are available. Presumably, older and previously infected birds would prove refractory.

Other species of *Microphallus* reported from mammalian hosts include *M. minus* by Ochi (1928) from rats, cats, dogs and man in Japan; *M. brevicaeca* by Africa and Garcia (1935) from man in the Philippine Islands; *M. gracilis* by Baer (1943) from *Neomys fodiens* taken in the environs of Geneva, Switzerland; *M. opacus* by Rausch (1947) from the opossum and the racoon; and *M. enhydrae* by Rausch and Locker (1951) from the arctic sea-otter. The metacercariae of *M. minus* and *M. opacus* occur in crustaceans and *M. opacus* matured in both poikilothermal and homothermal hosts. Since Rausch has shown that *M. opacus* can mature in opossums and racoons, the fact that the present specimens developed in mammals may be incidental and insignificant. *M. opacus*, like the present species, failed to infect chicks. It is entirely probable that the metacercariae in *Limulus* may mature in shore-birds. When the asexual stages and cercariae of the species are discovered, it will be possible to attempt infection of crustaceans and learn whether or not the parasite can use both arachnids and crustaceans as second intermediate hosts. Since the microphallid cercariae are very immature and the metacercariae undergo extensive development

in the second intermediate host, it must be more than a mere transfer host. In the present state of uncertainty concerning specificity in the genus *Microphallus* it seems inappropriate to assign the specimens described in this paper to *M. claviformis*, and they are designated as *M. limuli* n. sp. Actually the features employed by previous authors to distinguish between species of *Microphallus* are exceedingly variable and tenuous; and certain of the presently accepted species are probably identical. The experimental results of Rausch (1947) have thrown doubt on the validity of several species and, in correspondence, Cable has recently suggested that *M. nicolli* may be the same as *M. simile*.

In the paper mentioned earlier, Baer (1943) divided the family Microphallidae Travassos, 1921, into two families, Microphallidae and Maritrematidae, distinguished by the absence of a cirrus sac in the former and the presence of this organ in the latter. In the family Microphallidae, as restricted, he included the genera: *Microphallus* Ward, 1901; *Levinseniella* Stiles and Hassall in Ward, 1901; *Spelotrema* Jägerskiöld, 1901; *Monocaecum* Stafford, 1903; and *Spelophallus* Jägerskiöld, 1908. The family Maritrematidae, as conceived, contained the genera: *Maritrema* Nicoll, 1907; *Microphalloides* Yoshida, 1938; *Gynaecotyla* Yamaguti, 1939 (misspelled *Gynaecocotyla*,= *Cornucopula* Rankin, 1939); and *Pseudospelotrema* Yamaguti, 1939 (= *Maritreminoides* Rankin, 1939). It is to be noted that although Baer included five genera in the restricted family Microphallidae, in the same paper he suppressed *Spelotrema* and *Monocaecum* as identical with *Microphallus*. Moreover, he noted that *Spelophallus* differs from *Spelotrema* in only a single feature; that in the former genus the metraterm opens into the distal part of the genital atrium whereas in the latter it opens deeper in the atrium, near the base of the male papilla. *Spelophallus* is known by only a single species, *S. primus* Jägerskiöld, 1908. In these small, soft-bodied worms, where lack of skeletal support and contraction of different sets of muscles can produce extensive variation in the relations of such mobile parts, it is questionable whether *Spelophallus* has any true morphological basis. In my opinion, the differences described by Jägerskiöld can have no more than specific value. Accordingly, *Spelophallus* is suppressed as a generic concept and the species, *S. primus*, is transferred to *Microphallus* as *M. primus* (Jägerskiöld, 1908).

The proposal of Baer to divide the family Microphallidae was analyzed by Cable and Kuns (1951). They erected a new genus, *Carneophallus*, to contain *C. trilobatus* n. sp., and recognized a series of genera from *Microphalloides* and *Pseudospelotrema* to *Levinseniella* and *Carneophallus*, showing gradual reduction of the cirrus sac correlated with increased complexity in structure of the copulatory organs. Accordingly, they rejected the proposal of Baer, retained the genera with a cirrus sac in the family Microphallidae, and presented a scheme to illustrate probable evolution in the family. The argument of Cable and Kuns seems convincing and there appears to be no adequate basis for recognition of a family Maritrematidae.

Summary

Metacercariae from *Limulus polyphemus* developed to sexual maturity in white mice and golden hamsters. The worms are described as a new species, *Microphallus limuli*. Morphologically they are very similar to *M. claviformis* (Brandes, 1888), but bionomic features seem to preclude their allocation to that species. The genus

Microphallus is discussed, *Spelophallus* Jägerskiöld, 1908 is suppressed as a synonym and *S. primus* Jägerskiöld, 1908, the only known species, is transferred to *Microphallus* as *M. primus* (Jägerskiöld, 1908). In agreement with Cable and Kuns (1951), the family Maritrematidae Baer, 1943 is not accepted.

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NEW SPECIES OF SYNDESMIS AND A REVISION OF THE FAMILY UMAGILLIDAE WAHL, 1910 (TURBELLARIA: RHABDOCOELA)

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During the spring and summer of 1948 at the Scripps Institution of Oceanography, La Jolla, California, one of us (H. W. S.) examined various marine invertebrates for possible stages in the life-history of the mesozoan parasites of octopuses.[2] In dissections of the common sand-dollar, *Dendraster eccentricus,* several specimens of a rhabdocoel turbellarian were found. Whether they were originally in the intestine or the perivisceral cavity was not determined. Two specimens were fixed in Duboscq-Brasil solution, stained with Paracarmine, and mounted. Attempts at identification showed them to be different from all previously described species. Accordingly, preliminary sketches were made and the larger one was demounted, cut transversely in serial sections at 10 microns, and stained with haematoxylin and erythrosin. The presence of a large egg-capsule in each worm obscured certain structures in the whole mounts, and in the sectioned specimen, the adjacent structures were obviously distorted by pressure.

So far as we have been able to determine, this is the first rhabdocoel parasite to be described from the flattened, sand-dollar type of echinoid and the fifth species of the family Umagillidae reported from the Western Hemisphere. The morphology of the worms and review of the literature indicated their inclusion in the genus *Syndesmis.* Since the specimens could not be referred to any known species, they are described as a new species for which the name *Syndesmis dendrastrorum* is proposed.

The Genus Syndesmis

The genus *Syndesmis* was erected by Silliman (1881) but without specific designation, for a parasite of *Echinus sphaera* found at Roscoff, France. The account, without figures, was adequate to validate the generic name. What was probably the same species had been reported, but not named, by Geddes (1880) from the perivisceral fluid of the same species of sea-urchins at Roscoff. François (1886) reported *Syndesmis* from the intestine of *Strongylocentrotus lividus* and *Echinus acutus* at Banyuls sur Mer in the Mediterranean, emended the description of Silliman (misspelled Sillimann), and proposed the specific name, *S. echinorum.* Cuénot (1892) found *S. echinorum,* at least a score in each individual, in the coelomic cavity of *Echina sphaera* at Roscoff; he described the activities of the worms and stated that,

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like many other animals which live in ecological niches where the CO_2 is high and the O_2 tension is low, they contain haemoglobin in the lacunae of the mesenchyme. Russo (1895) gave a more complete account, illustrated with 36 figures, of the morphology of *S. echinorum* from *Sphaerechinus granularis* at Naples, with observations on the development and homologies of the genital structures. He stated that the parasites were located in the digestive tract rather than the coelom. Shipley (1901) described *S. echinorum* (misspelled *Syndesmus*) from ten specimens found in the alimentary canal and one from the perivisceral cavity of *Echinus esculentus* at Plymouth, England. He noted that the earlier accounts by Silliman, François, and Cuénot had promised complete descriptions, which apparently were never published. Shipley was aware of Russo's paper, but had not seen it. Von Graff (1903) stated that the genus *Syndesmis* contains a single, widely distributed species which he had found in the intestine of *Sphaerechinus granularis* at Bergen and of *Strongylocentrotus dröbacchensis* at Alexandrowsk. He described the characteristic movements of the worm and discussed the homologies of the female genital ducts, for which different terminology had been employed by previous authors.

Subsequent accounts have been given by other authors. Briot (1906) described, without figures, specimens which he found in the perivisceral cavity and intestine of *Strongylocentrotus lividus* and *Sphaerechinus granularis* at Marseilles, France. The worms were much larger than those reported previously and the measurements may have been made from much flattened specimens. He observed the exudation of red material from the mouths of pressed specimens and predicated that the pigment in the parasites came from the tissues of their hosts. Differences between worms from the two species of sea-urchins led to the belief that two distinct varieties were involved. In a series of papers, Wahl (1906, 1909, 1910a, 1910b) made important contributions to knowledge of the morphology and taxonomy of *Syndesmis*, although his work was overlooked by a number of later investigators. The monograph by von Graff (1913) on the rhabdocoel turbellarians listed *Syndesmis* with *S. echinorum* as the only species. Meixner (1923) discussed the origin and function of the egg-capsules and polar filaments of *Syndesmis* and other rhabdocoels. Westblad (1926a) described the excretory system of *S. echinorum*. Although most workers have regarded the syndesmid rhabdocoels of European sea-urchins as representatives of a single species, the accounts of the several authors list many differences in host, habitat, size, and internal structure. The figures of *S. echinorum* by Russo and by Shipley (Figs. 1, 2) show many differences, especially in shape and location of the testes and in the length and course of the genital ducts; consequently there appears some question whether *S. echinorum* designates a single species or a group of related forms. Indeed, Braun (1889) suggested that the descriptions of Silliman (1881) and François (1886) were concerned with different species.

A second species of *Syndesmis* was reported but not named by Powers (1936). About five per cent of the sea-urchins, *Centrechinus antillarum*, examined at the

The figures are copied or redrawn from original descriptions with special emphasis on the shape, size, location and relations of the testes, ovaries, and vitellaria, which we regard as features of essential taxonomic significance. Structures are similarly represented in all species and labelled in Figure 1. Abbreviations: br, brain; cg, cement glands; eg, egg-capsule in uterus; ga, common genital antrum; in, intestine; mo, mouth; ov, ovary; sd, common sperm duct; ts, testis; vt, vitellaria.

PLATE I

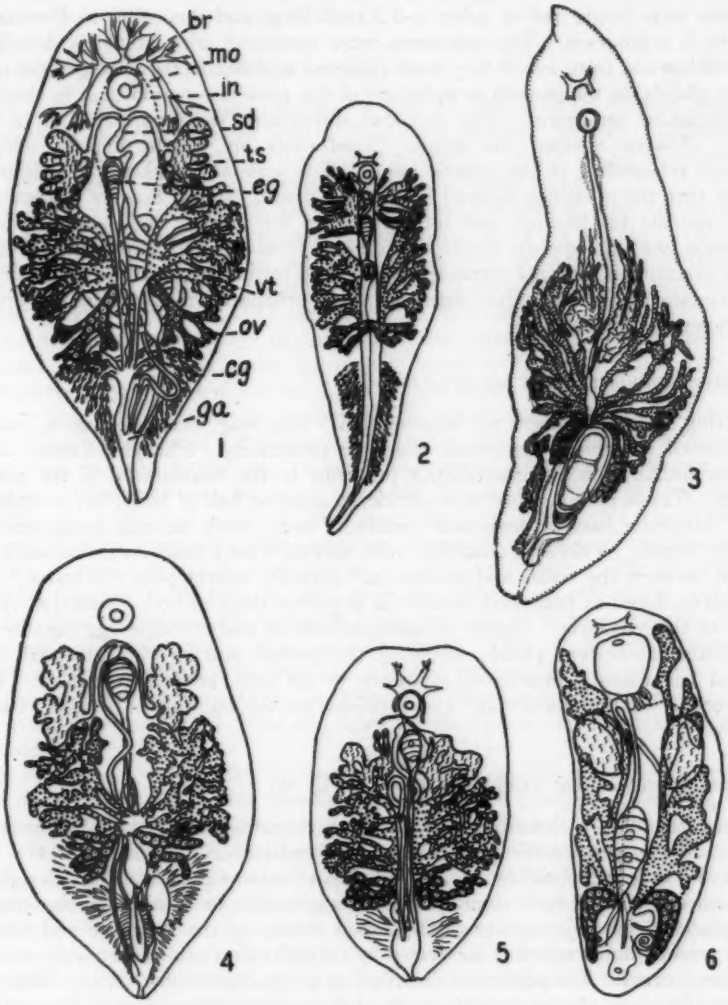


FIGURE 1. *Syndesmis echinorum* after Russo, 1895.
FIGURE 2. *Syndesmis echinorum* after Shipley, 1901.
FIGURE 3. *Syndesmis dendrastrorum* new species, original, camera lucida.
FIGURE 4. *Syndesmis franciscana* (Lehman, 1946) after Lehman.
FIGURE 5. *Syndesmis antillarum* new species, after Powers, 1936.
FIGURE 6. *Umagilla forskalensis* after Wahl, 1906.

Tortugas Laboratory, off the Florida Coast, were infected, each with five to sixteen worms in the coelomic cavity, attached to the outer wall of the intestine. The parasites were bright red in color, 1–1.5 mm. long, and according to Powers, related to *S. echinorum*. The specimens were compared with Shipley's description of *S. echinorum*, from which they were reported to differ in the arrangement of the cement glands, in the general morphology of the posterior region, and in details of the copulatory apparatus. The reported differences were portrayed in a text-figure. Powers labelled the figure "*Syndesmus* sp.," unfortunately adopting Shipley's misspelling of the generic name. The scale at the side of the figure indicates that the specimen figured was about 2 mm. long. Powers' account and figure validate his findings and for the species we propose the name *Syndesmis antillarum*, with the specific description derived from Powers' statement and figure. In the description we have employed the revised terminology of Dr. L. H. Hyman to designate the reproductive organs of the Turbellaria, rather than the original terminology of Powers.

Syndesmis antillarum new species (Fig. 5)

Living specimens bright red in color, 1–1.5 mm. long, oval to elongate, rounded or flattened anteriorly, somewhat attenuate posteriorly. Pharynx located in the anterior fourth of body, immediately posterior to the commissure of the nervous system. Testes paired, lobed to dendritic, in anterior half of body, but postpharyngeal. Muscular base of penis near middle of body, penis extends about one-third of body length. Vitellaria dendritic, with several (5–8) main stems on each side, located between the testes and ovaries and partially overlapping the testes. Ovaries paired, lobed or branched, located in posterior third of body, immediately posterior to the vitellaria. Uterus in anterior half of body; single egg-capsule with long filament; cement glands surround the genital antrum; bursal canal short; seminal bursa oval, immediately posterior to the oval, seminal receptacle. Habitat: coelom of the sea-urchin, *Centrechinus antillarum*, Dry Tortugas, Gulf of Mexico.

Syndesmis franciscana (Lehman, 1946) (Fig. 4)

Lehman (1946) described a parasitic turbellarian from the California sea-urchin, *Strongylocentrotus franciscanus* as *Syndisyrinx franciscanus*. He noted that *Syndesmis* and *Syndisyrinx* are parasites of echinoids and that in morphological features they are very similar, which suggested close relationship between the two genera. *Syndisyrinx* was distinguished chiefly on the structure and relations of the seminal bursa which is connected by cuticular ducts to the seminal receptacle and bursal canal. The penis was described as a cuticular hollow stylet. The name, *Syndisyrinx*, was chosen to indicate the presence of a "bursal valve," a cuticular structure seven microns in diameter and ten microns long, composed of the ends of the bursal and insemination canals and the sheath which surrounds them. These cuticular canals have been described for other members of the family although a minute sheath around their openings has not been reported. Lehman compared his specimens with the description of *Syndesmis echinorum* given by Russo (1895). On the basis of Meixner's (1926) re-description of that species, not cited by Leh-

man, Marcus (1949) suppressed *Syndisyrinx* as a synonym of *Syndesmis*. We concur in the opinion of Marcus and designate the species described by Lehman as *Syndesmis franciscana* (Lehman, 1946).

Syndesmis dendrastrorum new species (Fig. 3)

The turbellarian from sand-dollars of the Pacific coast is distinct from *S. echinorum* as described by the several European authors, from *S. antillarum* as figured by Powers, and from *S. franciscana* (Lehman, 1946). The description is based on laboratory notes and on two specimens, as noted earlier.

Living specimens colorless to gray, elongate oval to fusiform, flattened ventrally and slightly convex dorsally, widest at or just posterior to the middle of the body, 1–2.5 mm. in length, capable of much elongation with accompanying decrease in width. Two worms, flattened under one-half of a glass slide, fixed, stained and mounted, measured 1.58 by 0.42 mm. and 1.14 by 0.36 mm., respectively. Both were mature, each with a large egg-capsule in the uterus. The smaller specimen is shown in Figure 3. In it the egg-capsule measures 0.105 by 0.072 mm.; the capsule in the larger worm measured 0.198 by 0.08 mm. This worm was sectioned serially. The body is entirely covered with cilia, which measure about 3 microns in length. The epithelium is 4–6 microns in thickness, the cells roughly hexagonal in surface view, but cell boundaries are often indistinct. The nuclei measure about 4 microns in diameter and are often somewhat flattened. The cells rest upon a basement membrane and below it there is a thin layer of circular fibers, on the inner side of which there are small, separated, longitudinal fibers. The parenchyma is loose and vacuolate, traversed by many dorso-ventral fibers. The pharynx, of typical "doliiformis" structure, is situated in the anterior fourth of the body and measures about 50 microns in diameter. The intestine is narrow, tubular, dorsal in position; its walls consist of irregularly-shaped cells. It has no diverticula, does not extend anterior to the pharynx, and ends posteriorly a short distance anterior to the egg-capsule (about 45 microns in the sectioned specimen). The nerve commissure is located about two-thirds of the distance from the anterior end of the body to the pharynx.

The testes are paired, lobed to dendritic, in the posterior part of the anterior half of the body, a considerable distance posterior to the pharynx. The origins of the sperm ducts were not observed. The common sperm duct was traced forward from the base of the "penis" for a short distance between the posterior ends of the testes but could not be followed to its bifurcation into the sperm ducts. A small, more densely staining area, situated about two-thirds of the distance from the testes to the pharynx may represent a spermiducal vesicle. The muscular base of the penis is situated about three-fifths of the body length from the anterior end; the copulatory organ is a small, narrow, cuticular tube (about 2 microns in diameter), ventral in position, extending posteriad to open near the posterior end of the genital antrum. Vitellaria paired, dendritic glands, located principally in the middle third of the body; branches extend forward and laterad to the level of the middle of the testes, and backward, lateral to the ovaries, to the level of the genital antrum. The branches converge anterior to the ovaries; union of vitelline ducts with seminal receptacle not observed. Ovaries paired, lobed or branched, located in the posterior third of body, partially obscured by the large egg-capsule in the

uterus. Egg-capsule large, in posterior third of body, filament relatively short (less than twice capsule length), twisted and tangled. Sparse unicellular cement glands in the posterior fourth of body. Seminal bursa oval, immediately posterior to and confluent with oval seminal receptacle; bursal canal narrow, long; ovovitelline duct short; genital antrum opens at posterior end of body. The large egg-capsule exerted pressure on and distorted adjacent structures, preventing detailed study of the genital antrum and the ducts which open into it.

From the features listed, it appears proper to refer the specimens to the genus *Syndesmis*. They differ from *S. echinorum, S. antillarum* and *S. franciscana* in the greater distance between the pharynx and testes, in form of the intestine, greater extent of vitellaria, smaller pharynx, more posterior location of the uterus, sparser cement glands, larger size of egg-capsule and shorter and more delicate capsular filament. Its host belongs to a different order of the Echinoidea. These differences may be regarded as characteristic of *S. dendrastrorum*. The type specimen has been deposited in the U. S. National Museum, U.S.N.M. Helm. Coll. No. 47593.

The Family Umagillidae

Von Graff (1882) erected the family Vorticidae to contain certain rhabdocoel turbellarians and provisionally separated the parasitic from the free-living species. In a revision he (1903) dropped the subfamily divisions and included the parasitic genera, *Anoplodium* Schneider, 1858, *Syndesmis* Silliman, 1881, and *Collastoma* Dörler, 1900, with several free-living genera in the family Vorticidae. With the acceptance of *Dalyellia* as the valid generic name for the species formerly included in *Vortex*, he (1905) proposed the family name Dalyelliidae with two subfamilies: Dalyelliinae with paired ovaries and Graffillinae with unpaired ovary; the latter he (1908) raised to family rank. The genus *Anoplodium* was included in the family Dalyelliidae; the genera *Syndesmis, Collastoma* and *Umagilla* in the family Graffillidae.

Wahl (1906) published the first of a series of papers on the parasitic turbellarians. He studied the morphology of members of the genera *Anoplodium, Graffilla, Paravortex, Collastoma,* and erected a new genus, *Umagilla.* Wahl (1909) published further studies on *Umagilla* and *Syndesmis,* and showed that the basis of von Graff's (1905) arrangement was untenable. He (1910a) presented further evidence, based especially on the female genital ducts, to show that *Umagilla, Syndesmis, Anoplodium* and *Collastoma* constitute a related group, distinct from the other genera included in von Graff's families Dalyelliidae and Graffillidae. The genus *Umagilla* was regarded as the most primitive and characteristic member of this group and was named type of a new family, Umagillidae. In the family, "Die als Receptaculum seminis fungierende Vereinigungsstelle von Keim- und Dotterstöcken (bezw. von deren Ausführungsgängen) ist mit dem Atrium genitale durch einen Ductus communis und eine Vagina verbunden." Two subfamilies were recognized: Umagillinae with paired testes and Collastominae with a single, median testis. The first subfamily included *Umagilla, Syndesmis* and *Anoplodium;* the second only the genus *Collastoma.* Wahl (1910b) made further studies on the genus *Collastoma.*

Von Graff (1913) accepted the systematic arrangement proposed by Wahl (1910a), in which the family Graffillidae was suppressed and the genera, other than

those included in the Umagillidae, were transferred to the family Dalyelliidae. However, without explanation and in contravention of the Rules of Nomenclature, he proposed the family Anoplodiidae and the subfamily Anoplodiinae, listing Umagillidae and Umagillinae as synonyms. Six valid and five doubtful species were included in the family.

Since the monograph by von Graff (1913), many additions have been made to knowledge of the family, the number of genera and species has trebled, but there has been no detailed systematic account of the group. Only four families of the Rhabdocoela are completely parasitic and the family Umagillidae is by far the largest; in fact, this family constitutes the largest parasitic group in the entire class Turbellaria. All members of the Umagillidae are parasitic, living in the intestine or body cavity of echinoderms or in one instance, in the alimentary tract of sipunculid worms. Ward (1933) noted that, "both by virtue of numbers of species parasitic in echinoderms and of frequence in those hosts the Turbellaria may rightly be designated as the major group of echinoderm parasites." Steinmann and Bresslau (1913) adopted von Graff's family and subfamily names Anoplodiidae and Anoplodiinae in their monograph on "Die Strudelwürmer". Beklemicheff (1916) erected two new genera, *Desmote* and *Pterastericola,* to contain two new species, the first from a crinoid and the second from a sea-star. These are the first parasitic turbellarians reported from their respective echinoderm hosts and both genera were included in the family Dalyellidae. In a series of papers Meixner (1923, 1924a, 1924b, 1926) discussed the morphology and classification of the rhabdocoel turbellarians; he adopted the family name Anoplodiidae and suggested a new family Pterastericolidae. Kaburaki (1925) proposed a new genus, *Bicladus,* for a new species, *B. metacrini,* from *Metacrinus rotundus.* Steinböck (1925 transferred the species to *Desmote* and suppressed the generic name *Bicladus,* but Poche (1925) accepted *Bicladus* as a valid genus and, in fact, designated it as type of a new family. Subsequent papers in which this worm has been mentioned have tacitly agreed with Steinböck's disposal of the species and have not cited Poche's paper.

Faust (1924) reported a supposed holostome trematode from the holothurian, *Actionopyga mauritiana,* from the Andaman Sea. He named the parasite *Cleistogamia holothuriana* and later (1927) published a more complete account. On the basis of Faust's description, Bosma (1931) erected a new family, Cleistogamiidae, in the trematode superfamily Strigeoidea. Influenced by Faust's (1927) paper on *Cleistogamia,* Khalil and Azim (1937) described worms from holothurians of the Red Sea as trematodes under the designation *Loutfia loutfia* n. g., n. sp. They considered *Loutfia* and *Cleistogamia* to represent a new family which, unaware of Bosma's paper, they named Cleistogamidae. Later, Khalil (1937) studied living specimens and, in a footnote to the above paper, reported that the supposed trematode was a rhabdocoel turbellarian belonging to the family Anoplodiidae. Khalil (1938) redescribed the species as *Cleistogamia loutfia.* Baer (1938) independently had studied specimens of *Cleistogamia holothuriana* deposited by Faust in the Indian Museum. He showed that Faust had confused anterior and posterior ends, misinterpreted the internal anatomy, and that a complete new description was necessary. In a postscript, Baer referred to the papers by Khalil and Azim (1937) and Khalil (1937) and pointed out that the vitellaria are so different in *Cleistogmia* and *Loutfia*

that it is unlikely that the two are identical. Marcus (1949) tacitly suppressed *Loutfia* as a synonym of *Anoplodiera* in naming *L. loutfia* as *Anoplodiera loutfia* (Khalil and Azim).

Bock (1926) described a new species, *Anoplodium stichopi,* from the body cavity of *Stichopus tremulus;* for it he proposed a new subgenus, *Anoplodiella.* He mentioned, but did not describe, another new species from the same host which he referred to the genus *Umagilla.* Westblad (1926a) described the excretory system of *Syndesmis echinorum* and also (1926b) reported five species, all as members of the family Umagillidae, from *Stichopus tremulus.* One of the five, *Anoplodium stichopi,* had been described by Bock (1926); two others, *Anoplodiera voluta* and *Wahlia macrostylifera,* representing new genera, were described by Westblad (1930); the others were listed as *Umagilla elegans* Bock and *Meara stichopi* Bock. Westblad (1949) described *Meara stichopi* and showed that it is a member of the Turbellaria archoophora, suborder Nemertodermatida. Westblad (1930) discussed the female genital ducts and regarded the "female atrium" of earlier workers as a "secondary uterus"; he reported that *Umagilla elegans* may contain as many as 26 mature egg-capsules in its large "sekundären Uterus." Ozaki (1932) described *Anoplodium mediale* n. sp., from the body cavity, and *Xenometra arbora* n. g., n. sp., from the intestine of *Stichopus japonicus.* Bresslau (1933) reverted to the use of the family name Anoplodiidae, although Westblad had used the correct name Umagillidae, and the subfamilies were dropped. Eight genera and twelve species were listed and, without discussion, the subgenus *Anoplodiella* was raised to generic rank. In agreement with Steinböck (1925), the new species described by Kaburaki (1925) was listed as *Desmote metacrini.* Marcus (1949) in a monograph on the Turbellaria of Brazil, employed the invalid name Anoplodiidae on the erroneous assumption that the family name must be derived from that of the oldest genus. He noted that the name *Xenometra* Ozaki, 1932 was preoccupied by *Xenometra* Emery, 1917 and for a replacement he proposed the generic name *Ozametra.* He described a new species, *Anoplodium ovelinae* and suppressed *Anoplodiella* as a synonym of *Anoplodium.*

Four parasitic rhabdocoels of the family Umagillidae have been reported previously from the Western Hemisphere. The first was described by Meserve (1934) as *Macrogynium ovalis* n. g., n. sp., from *Stichopus* sp. at Bermuda. The account is inadequate, the terminology employed differs from that customarily used in describing rhabdocoels, and there is no comparison with other parasitic turbellarians. Since there is no differential diagnosis of either genus or species, as required by the action of the XIth International Congress of Zoology, the validity of the names proposed by Meserve is questionable. However, the testis and female genitalia, as described, exclude the species from other genera in the family. Marcus (1949) recognized *M. ovalis* as a valid name. The second endoparasitic rhabdocoel from the Western Hemisphere was reported by Powers (1936) as *"Syndesmus* sp." from *Centrechinus antillarum.* His account and figure provide the basis for the description in which we have named the species *Syndesmis antillarum.* The last genus to be added to the family and the third species from the Western Hemisphere was described by Lehman (1946) from the intestine of the California sea-urchin, *Strongylocentrotus franciscanus,* as *Syndisyrinx franciscanus* but, as noted earlier, Marcus (1949) suppressed the name *Syndisyrinx* as a synonym of *Syndesmis* and

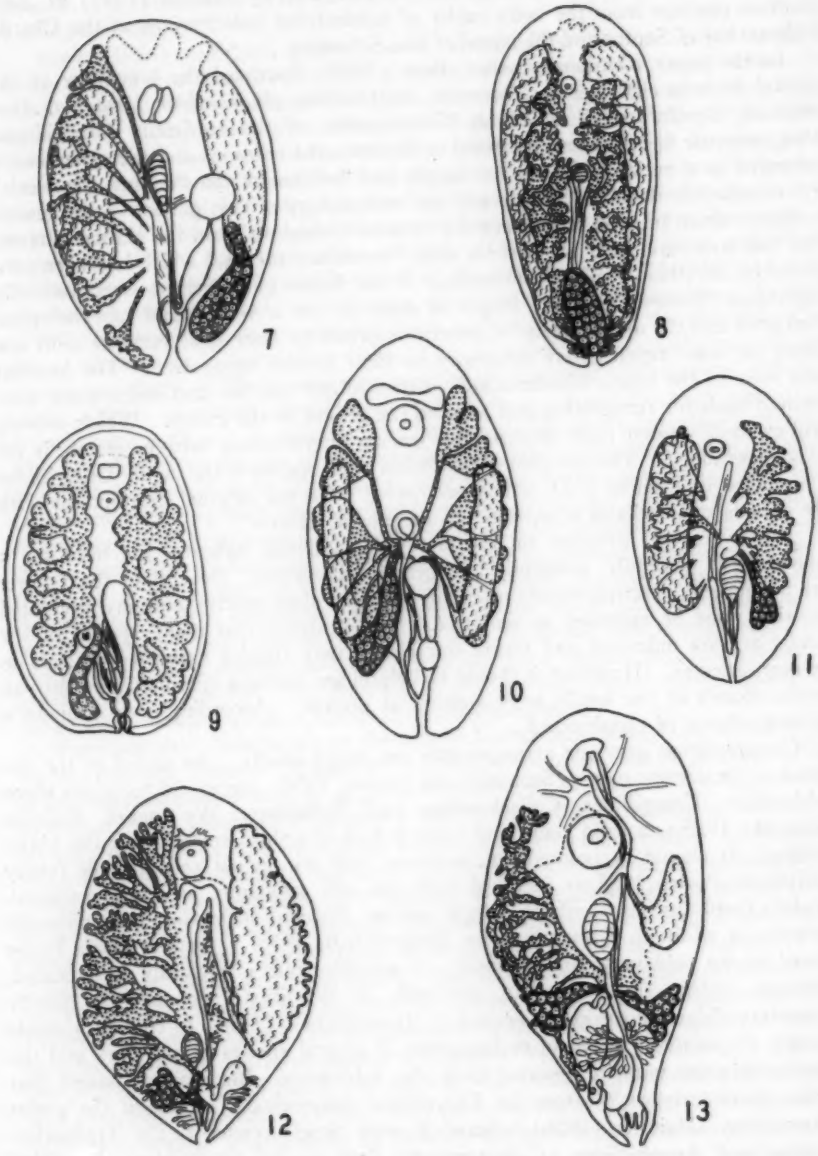
we have designated the species, *Syndesmis franciscana* (Lehman, 1946). The fourth species from the Western Hemisphere was described by Marcus (1949) as *Anoplodium evelinae* from the body cavity of unidentified holothurians at the Ilha da Palmas, bay of Santos and the island of São Sebastião.

In the paper mentioned earlier, Baer (1938) discussed the homology of the genital ducts in eight genera, *Desmote, Anoplodiella, Anoplodiera, Umagilla, Anoplodium, Syndesmis, Wahlia* and *Cleistogamia,* of the subfamily Umagillinae. Diagrammatic figures were presented to illustrate the relations and the genera were arranged in a series based on the length and location of the "ductus communis" (= ovovitelline duct) and the length and morphology of that portion of the female complex which intervenes between the "ductus communis" and the genital antrum. For this structure, Baer adopted the term "secondary uterus," which had been proposed by Westblad (1930). Homology of the female genital ducts is undoubtedly significant; however, relative length of ducts is not a very sound morphological character and the arrangement of genera proposed by Baer is at variance with one based on other reproductive structures as Baer himself recognized. The location and form of the testes, vitellaria and ovaries afford another and much more convenient basis for recognizing and relating the genera in the group. Which criteria will eventually prove more important in tracing interrelations between genera is yet to be determined. The morphology of all described species of the family Umagillidae is depicted in Figures 1–23, which are copied from the original descriptions with the gonads and vitellaria represented as diagnostic features.

Review of the literature on the family Umagillidae indicates the need for a re-statement of family, subfamily and generic characters. Several of the reports are incomplete, descriptions are often vague rather than precise, and any attempted revision must be regarded as tentative. It is probable that many species of the family are yet unknown and future discoveries may require extensive changes in present concepts. However, it should be helpful and perhaps stimulating to have an outline-sketch of the family as it appears at present. Accordingly we propose a revised scheme of classification.

Changes from previous arrangements are noted briefly. As stated in the abstract of the present paper (Stunkard and Corliss, 1950), we would recognize three subfamilies: Umagillinae, Collastominae, and Bicladinae. We regard *Bicladus* Kaburaki, 1925 as a valid genus and name it type of a third subfamily in the Umagillidae. It should be pointed out, however, that we do not consider the family Bicladidae Poche, 1925 as identical with the new subfamily we have proposed. Poche's family included only the single species, *Bicladus metacrini;* our subfamily includes in addition, *Desmote vorax* Beklemicheff, 1916, a species which Poche placed in the subfamily Anoplodiinae. A possible fourth subfamily is indicated. Meixner (1926), in a footnote and with no definition, proposed the family Pterastericolidae for the genus erected by Beklemicheff (1916) to contain a single species, *P. fedotovi.* It is an endoparasite of several species of sea-stars and the only turbellarian parasite reported from the Asteroidea. Beklemicheff stated that it has characteristics "de tous les Dalyelliidae en général," especially the genus *Phaenocora.* Meixner (1926) believed it more closely related to the Typhlorhynchidae and Anoplodiidae (Collastominae) than to the Graffillidae. Westblad

PLATE II



(1926a) included the genus in the family Dalyelliidae. A diagnosis of the family Pterastericolidae was given by Bresslau (1933). The species in question (Fig. 14) is very similar to certain of the Umagillidae and it appears that the inclusion of *Pterastericola* as type of a subfamily Pterastericolinae in the Umagillidae should be considered seriously. Apparently, only the more anterior position of the genital pore contravenes the family characters listed below. In other and more minor changes, we reduce *Anoplodiella* Bock to synonymy with *Anoplodium*, *Syndisyrinx* Lehman to synonymy with *Syndesmis*, and pending further information, *Loutfia* Khalil and Azim is suppressed as a synonym of *Cleistogamia*.

The species of Umagillidae, recognized as valid, are arranged in a key; in addition, eight others have at one time or another been placed in the genus *Anoplodium*, either as valid or questionable species. In our opinion, they can not be recognized in the family Umagillidae. The list comprises:

1. *A.* ? *schneideri* Semper, 1868; the specimens were from *Stichopus variegatus* and *Mülleria lecanora* in the Philippine Islands. There was no description or figure and the name is invalid.
2. *A.* ? *clypeasteris* v. Graff, 1882; the name was proposed for red planarians from the surface of *Spatangus* sp., reported by Moseley (1874); Cuénot (1892) suggested a possible relationship to *Syndesmis* since both contained haemoglobin.
3. *A.* ? *mytili* Levinsen, 1879; an oculate species transferred to *Graffilla* by von Graff (1882).
4. *A. myriotrochi* v. Graff, 1882; reported but not described or named by Danielssen and Koren (1880) as a "smaa rødlige Planarier" from the intestine of *Myriotrochus rinkii* at Spitzbergen.
5. *A. pusillum* Monticelli, 1892; reported from the body cavity of *Holothuria poli* at Naples. Von Graff (1913) listed it as a synonym of *A. parasita*.
6. *A. graffi* ? Monticelli, 1892; from *Holothuria impatiens* at Naples; von Graff (1913) listed it as an uncertain species.
7. Vorticide (Gen. ? sp. ?) Monticelli, 1892; found in *Cucumaria planci* at Naples; no description; listed as a possible *Anoplodium* by von Graff (1903).
8. *A. chirodotae* Sabussow, 1900; from the body cavity of *Chirodota pellucida;* an oculate species which von Graff (1913) excluded from *Anoplodium*.

FAMILY UMAGILLIDAE WAHL, 1910 (SYN. ANOPLODIIDAE VON GRAFF, 1913).

Endoparasitic Rhabdocoela with no rostrum, rhabdites, dermal or adhesive glands, or eyes. Ventral "pharynx doliiformis," situated in anterior third of body; genital pore located posteriorly, single with one exception (*Desmote*). Intestine

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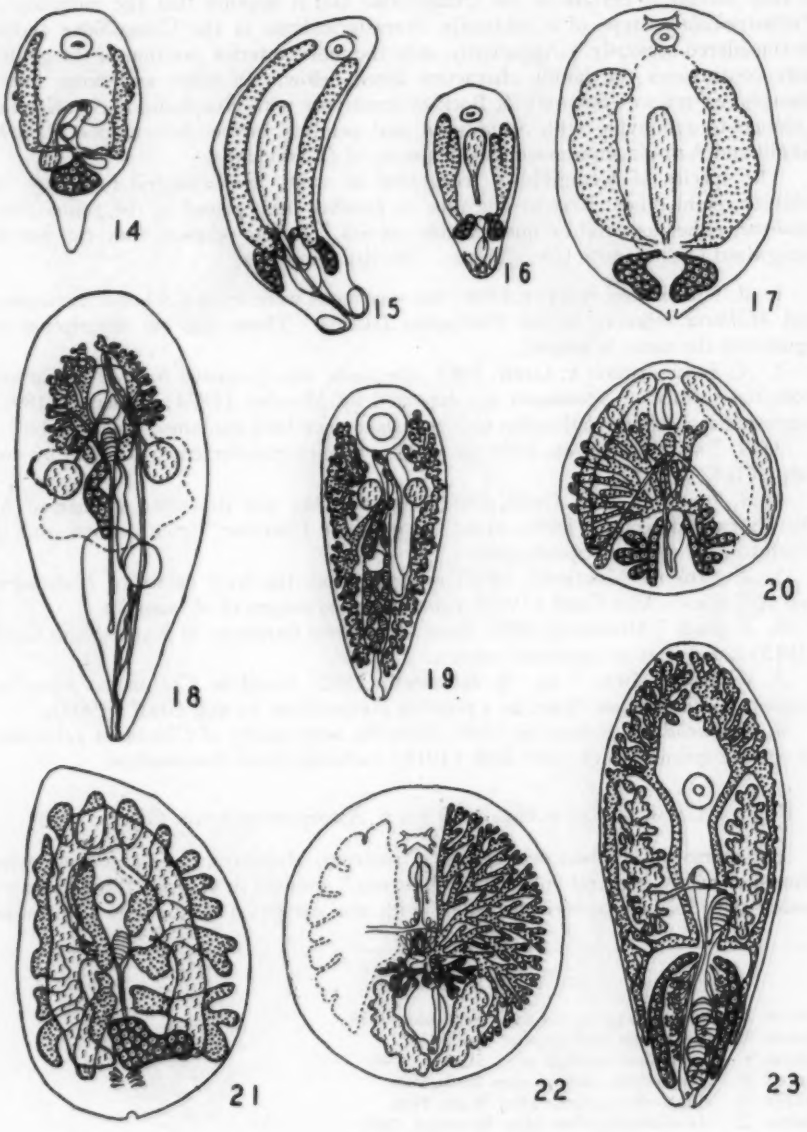
PLATE II

FIGURE 7. *Anoplodium parasita* after Schneider, 1858.
FIGURE 8. *Anoplodium mediale* after Ozaki, 1932.
FIGURE 9. *Anoplodium evelinae* after Marcus, 1949.
FIGURE 10. *Anoplodium stichopi* after Bock, 1926.
FIGURE 11. *Anoplodium gracile* after Wahl, 1906.
FIGURE 12. *Anoplodiera voluta* after Westblad, 1930.
FIGURE 13. *Wahlia macrostylifera* after Westblad, 1930.

PLATE III



fairly compact, not sharply delimited, rarely (*Desmote, Bicladus*) in two extensions. Brain well developed. Testes paired or unpaired, for the most part posterior to the pharynx; spermiducal vesicle present, male copulatory organ with or without cuticularized structures. Ovaries single or paired, posterior to testes and vitellaria, a single exception (*Bicladus*). Vitellaria generally extensive, commonly branched and lateral, in one case (*Cleistogamia*) peribuccal. Uterus formed as a protrusion of the common genital antrum, rarely (adult *Desmote*) an independent structure. Bursal canal, ovovitelline duct, and seminal receptacle always present. Parasites in the alimentary canal or body cavity of echinoderms or (*Collastoma*) sipunculids.

Subfamily UMAGILLINAE Wahl, 1910 (syn. ANOPLODIINAE v. Graff, 1913).

Body oval to elongate; intestine saccate to tubular; testes paired (fused anteriorly in *Macrogynium* ?); vitellaria follicular and branched; ovovitelline duct enters common genital antrum ventral or anterior to the common sperm duct. Parasites of echinoderms, classes Echinoidea and Holothurioidea. Type genus: *Umagilla* Wahl, 1906; includes also *Anoplodium* Schneider, 1858; *Syndesmis* Silliman, 1881; *Cleistogamia* Faust, 1924 (as redescribed by Baer, 1938); *Wahlia* Westblad, 1930; *Anoplodiera* Westblad, 1930; *Ozametra* Marcus, 1949 (= *Xenometra* Ozaki, 1932); and *Macrogynium* Meserve, 1934.

Subfamily COLLASTOMINAE Wahl, 1910.

Body oval to elongate; intestine saccate; testis single, median; vitellaria smooth or slightly branched; ovovitelline duct enters the common genital antrum dorsal or posterior to the common sperm duct. Parasites of sipunculid worms. Type and only genus: *Collastoma* Dörler, 1900.

Subfamily BICLADINAE Stunkard and Corliss, 1950.

Body nearly circular in outline; intestine H-shaped, a saccate lobe on either side of body; testes paired, typically posterior but may extend anteriad in *Desmote;* vitellaria extensively branched; genital pore or pores ventral, near posterior end of body; ovovitelline duct very short, enters directly into the uterus. Parasites of echinoderms (Crinoidea). Type genus: *Bicladus* Kaburaki, 1925; includes also *Desmote* Beklemicheff, 1916.

PLATE III

FIGURE 14. *Pterastericola fedotovi* after Beklemicheff, 1916.
FIGURE 15. *Collastoma minutum* after Wahl, 1906.
FIGURE 16. *Collastoma eremitae* after Beklemicheff, 1916.
FIGURE 17. *Collastoma monorchis* after Dörler, 1900.
FIGURE 18. *Cleistogamia holothuriana* after Baer, 1938.
FIGURE 19. *Cleistogamia loutfia* (Khalil and Azim, 1937) after Khalil and Azim.
FIGURE 20. *Desmote vorax* after Beklemicheff, 1916.
FIGURE 21. *Macrogynium ovalis* after Meserve, 1934.
FIGURE 22. *Bicladus metacrini* after Kaburaki, 1925.
FIGURE 23. *Ozametra arbora* (Ozaki, 1932) after Ozaki, 1932.

KEY TO THE FAMILY UMAGILLIDAE

Subfamily UMAGILLINAE Wahl, 1910:

1. (16) With paired ovaries 2
2. (3) Spermiducal vesicle anterior to brain, venter not ciliated .....*Wahlia* Westblad, 1930
   One species, ...*W. macrostylifera* Westblad, 1930
3. (2) Spermiducal vesicle posterior to brain, body covered with cilia, ...4
4. (7) Pharynx large, near anterior end of body, ...*Umagilla*, Wahl, 1906 5
5. (6) Single egg in uterus, ...*U. forskalensis* Wahl, 1906
6. (5) Many eggs in large, "secondary uterus".....*U. elegans* Bock in Westblad, 1926
7. (4) Pharynx small, more posterior in position, ...8
8. (9) Vitellaria extend anterior to testes,.....*Ozametra* Marcus, 1949
   One species,.....*O. arbora* (Ozaki, 1932)
9. (8) Vitellaria posterior to testes, ...*Syndesmis* Silliman, 1881 10
10. (15) Uterus extends forward to anterior third of body, mature egg-capsule at testicular level, ...11
11. (12) Vagina long, seminal bursa confluent with seminal receptacle, ...*S. echinorum* François, 1886
12. (11) Vagina short, independent seminal bursa,.....13
13. (14) Specimens 2–3 mm. long, from *Strongylocentrotus*, Pacific ocean, ...*S. franciscana* (Lehman, 1946)
14. (13) Specimens 1–1.5 mm. long, from *Centrechinus*, Gulf of Mexico,....*S. antillarum* n. sp.
15. (10) Uterus in posterior third of body, extends forward only to ovarian level, ...*S. dendrastrorum* n. sp.
16. (1) With single ovary,.....17
17. (18) Ventral surface not ciliated, ...*Anoplodiera* Westblad, 1930
    One species, ...*A. voluta* Westblad, 1930
18. (17) Body entirely covered with cilia,.....19
19. (20) Testes united anteriorly (?), ...*Macrogynium* Meserve, 1934
    One species, ...*M. ovalis* Meserve, 1934
20. (19) Testes separate, ... 21
21. (24) Pharynx very large; testes small, near pharynx.....*Cleistogamia* Faust, 1924 22
22. (23) Vitellaria peribuccal; single egg in uterus, ...*C. holothuriana* Faust, 1924
23. (22) Vitellaria extend along sides of body; many eggs in uterus, ...*C. loutfia* (Khalil and Azim, 1937)
24. (21) Pharynx small; testes large, extend from level of pharynx to ovary, ...*Anoplodium* A. Schneider, 1858 25
25. (30) Anterior portion of vagina enlarged as seminal bursa, ...
26. (29) Vitellaria extend almost length of body, ...27
27. (28) Ovary on left side, ...*A. parasita* Schneider, 1858
28. (27) Ovary on right side, ...*A. evelinae* Marcus, 1949
29. (26) Vitellaria shorter, about same extent as the testes, ...*A. gracile*, Wahl, 1906
30. (25) No seminal bursa, ...
31. (32) Testes small, in middle third of body; ovary lateral, ...*A. stichopi* Bock, 1926
32. (31) Testes large, nearly as long as body; ovary median, ...*A. mediale* Ozaki, 1932

Subfamily COLLASTOMINAE Wahl, 1910:

One genus, *Collastoma* Dörler, 1900, with three species,

1. (2) Ejaculatory duct muscular, not chitinized, ...*C. monorchis* Dörler, 1900
2. (1) Male copulatory organ a long chitinized tubule 3
3. (4) Vitellaria simple elongate glands, ...*C. minutum* Wahl, 1906
4. (3) Vitellaria divided into 4–6 parallel branches in mature individuals, ...*C. eremitae*, Beklemicheff, 1916

Subfamily BICLADINAE Stunkard and Corliss, 1950:

1. (2) Testes posterior to ovaries; single genital pore, ...*Bicladus* Kaburaki, 1925
   One species, ...*B. metacrini* Kaburaki, 1925
2. (1) Testes anterior and lateral to ovaries; two separate genital pores in mature individuals, ...*Desmote* Beklemicheff, 1916
   One species, ...*D. vorax* Beklemicheff, 1916

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RE-FERTILIZATION OF THE FERTILIZED EGGS OF THE SEA URCHIN [1]

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It has up to now been believed that spermatozoa cannot penetrate into the fertilized eggs of the sea urchin even if the fertilization membrane has been removed. Loeb says (1916, p. 85) on this point: "It is well known that if an egg is once fertilized it becomes impermeable for other spermatozoa." In regard to the same point, Lillie (1919, p. 161) states: "The fertilized egg does not react to fresh spermatozoa, and these do not enter it . . . and it has been repeatedly shown that removal of the fertilization membrane does not render the egg more susceptible to superimposed insemination." Just (1939, p. 190) also expresses a similar belief in his publication as follows: "With fertilization, they pass beyond the fertilizable condition. I know of only one report (Morgan, 1895), based on insufficient evidence, which claims that fertilized eggs can be re-fertilized. In my experience, eggs having been fertilized lose capacity for fertilization for neither they nor their fragments can be fertilized again."

The present author has found that when the fertilized eggs have been washed with Ca-Mg-free media after removal of the fertilization membrane, they again become permeable to other spermatozoa. In 1947, some results of the experiments were preliminarily reported by him in Japanese. More recently he has found that spermatozoa can enter the fertilized eggs without removal of the fertilization membrane if the eggs are treated with Ca-Mg-free media before the hardening of the membrane. Since these results seem to pose certain interesting problems, it should be worthwhile to report them somewhat in detail.

Material and Methods

The following sea urchins were employed: *Strongylocentrotus pulcherrimus, Heliocidaris crassispina, Temnopleurus toreumaticus* and *Pseudocentrotus depressus.* The egg of *Strongylocentrotus pulcherrimus* proved the best one for the investigation since the fertilization membrane can be easily removed and the clear protoplasm renders mitotic asters easily visible in the living egg.

The first insemination was performed in the usual manner. In the first series of experiments, the fertilization membrane was removed by repeatedly sucking and blowing the eggs with a pipette which had a diameter slightly exceeding that of the egg. The denuded eggs were then exposed to a Ca-Mg-free solution. After immersion for several minutes, the eggs were put back into the normal sea water and then the second insemination was carried out.

In another series of experiments, fertilized eggs were put into a Ca-Mg-free

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solution before hardening of the fertilization membrane. On their return to sea water, superimposed insemination was performed without removal of the membrane. Whether or not spermatozoa penetrated into the eggs at the time of the second insemination and afterwards took part in the cleavage was examined before and after the first or second cleavage. The re-fertilized eggs showed abnormal mitotic figures and cleavages characteristic of the polyspermic eggs.

Experimental Results

Re-fertilization of the eggs deprived of the fertilization membrane

A. Experiments in Strongylocentrotus pulcherrimus

The eggs of *Strongylocentrotus* which had been normally fertilized were deprived of the fertilization membrane mechanically. This procedure was carried out two minutes after insemination, just after the membrane had been raised and before

Table I

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed with Ca-Mg-free sea water (6° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	1%	99%	1%
10^{-3}	0	8	92	
10^{-4}	0	82	18	
10^{-5}	1	98	1	

it had hardened. A fresh sperm suspension was added to the denuded eggs thus obtained and the development of these eggs was observed. It was found that in these eggs the cleavage was quite normal and polyspermic division was as infrequent as in the control. The result of this experiment indicates that the denuded eggs in that condition cannot be fertilized again, and coincides perfectly with those of the earlier workers.

In the next experiments, the denuded eggs obtained by the above-mentioned procedure were washed with Ca-Mg-free sea water for 5 minutes, and were put back into the normal sea water. The second insemination was carried out in watch glasses. The undiluted sperm, taken directly from the testes of freshly opened males, was taken as a standard and various concentrations of sperm were made by ten-fold serial dilutions. To 4.5 cc. of the egg suspension was added 0.5 cc. of a sperm suspension. In all the experiments, parallel controls were run with eggs which were not inseminated again after washing with Ca-Mg-free sea water. The eggs were examined after two or three hours. One of the results of the experiments is shown in Table I.

It can be seen that more than 90 per cent of the eggs showed polyspermic features at the first cleavage if the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}. Some of such eggs showed the irregular division

characteristic of polyspermic eggs while the other heavily polyspermic eggs failed to divide, showing numerous asters in the protoplasm. In the control, polyspermic eggs were very few. The result indicates that spermatozoa penetrated into the previously fertilized eggs at the time of superimposed insemination and afterwards took part in the formation of the mitotic figure. The present author wishes to call this phenomenon "re-fertilization," since spermatozoa not only enter fertilized eggs but also participate in the mitotic process of the eggs.

A number of similar experiments were also conducted using a molar urea solution (pH 7.0) instead of Ca-Mg-free sea water. It was found that a molar urea solution was more effective than Ca-Mg-free sea water. One of the results is shown in Table II.

When the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}, 100 per cent of the eggs were re-fertilized and heavy polyspermy occurred in high percentages. Such heavily polyspermic eggs formed many asters in the protoplasm, but they did not cleave. It appears that the formation of too

TABLE II

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed in a molar urea solution (8° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	0%	100%	1%
10^{-3}	0	0	100	
10^{-4}	0	32	68	
10^{-5}	1	96	3	

many asters caused the failure of cytoplasmic division. It is also seen in Tables I and II that re-fertilization was hard to induce when the concentration of sperm in the second insemination was 10^{-5}. This is a noteworthy fact since in the usual experiments fertilization can occur with a much lower concentration of sperm.

In Figure 1, microphotographs of re-fertilized eggs and controls are shown. In normal eggs polyspermy is not found (Fig. 1a). The eggs which were inseminated again after the removal of the fertilization membrane in normal sea water cleave normally (Fig. 1b). This means that re-fertilization has not taken place. In the eggs deprived of the membrane after fertilization and washed with urea solution, no irregularity of cleavage is found, though the blastomeres are spherical and well separated from each other owing to the lack of the hyaline layer (Fig. 1c). The eggs deprived of the fertilization membrane will, when washed with urea solution and inseminated again, become polyspermic (Fig. 1d). It is clear that re-fertilization has occurred in these eggs.

In a group of experiments, the removal of the fertilization membrane was done in a Ca-Mg-free medium. This method was found to be simpler and more convenient than that of the above-mentioned experiments, because the fertilization membrane was easily removable and both the removal of the membrane and washing with Ca-Mg-free medium were done at the same time. It is a well-known fact that Ca-ion is

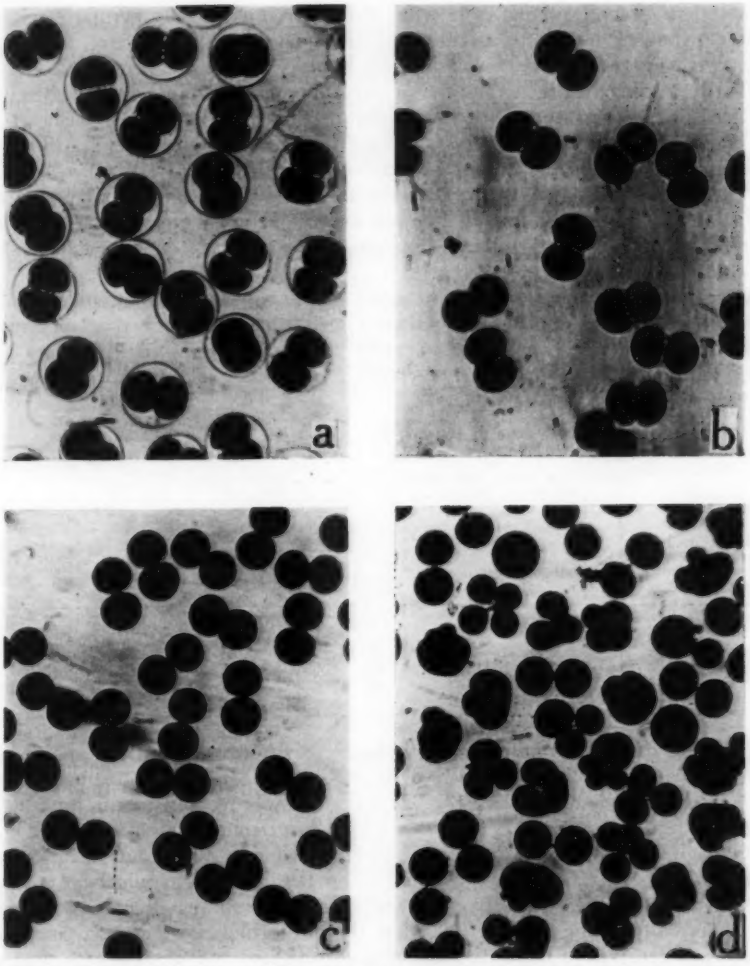

FIGURE 1. Re-fertilization of Strongylocentrotus eggs. The photographs were all taken of living eggs after the first cleavage. a. Normal eggs (control). b. Eggs which were inseminated again after removal of the fertilization membranes in normal sea water. c. Eggs which were deprived of the membrane and washed with urea solution, but not re-inseminated. d. Eggs which were deprived of the membrane, washed with urea solution and re-inseminated.

indispensable for hardening of the fertilization membrane. If the fertilized eggs are transferred into a Ca-Mg- free medium before the membrane has hardened, the membrane will not become tough and is elevated very high so that its removal is easy. One of the results is shown in Table III. It is evident from this that re-fertilization can be induced by this method.

TABLE III

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in a Ca-Mg-free medium (9° C.)

Medium in which membrane was removed	Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10^{-2}	1%	1%	98%	1%
	10^{-3}	0	5	95	
	10^{-4}	0	80	20	
	10^{-5}	1	98	1	
Urea solution (1 *M*)	10^{-2}	0	0	100	0
	10^{-3}	0	1	99	
	10^{-4}	0	49	51	
	10^{-5}	0	94	6	

In the above-mentioned experiments, superimposed insemination was carried out in sea water immediately after washing with a Ca-Mg-free medium. It was of some importance to ascertain whether or not re-fertilization could take place when superimposed insemination was induced at a later stage of cell division. Some experiments were carried out accordingly. The fertilized eggs were put into a Ca-Mg-free medium before the fertilization membrane had hardened, and the membrane was mechanically removed. After that they were put back into sea water in a few minutes and fresh sperm suspensions were added again at various stages of the early development of the eggs. The results are summarized in Table IV.

There is clearly indicated, by the inspection of these data, the possibility of re-fertilization taking place at any stage of the first cleavage. If the eggs are re-

TABLE IV

Re-fertilization of once-fertilized eggs at various stages of development. The membranes were removed in urea solution immediately after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3}, temp.: 13° C.)

Time from 1st insemination to 2nd insemination	Stage when observed	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs
6 m.	After 1st cleavage	1%	7%	92%
25 m.	After 1st cleavage	3	2	95
50 m.	After 1st cleavage	2	3	95
1 hr. 5 m.	After 1st cleavage	3	93	4
	After 2nd cleavage	2	8	90
2 hrs. (2-cell stage)	After 2nd cleavage	2	5	93
Control	After 2nd cleavage	2	98	0

fertilized before the anaphase, the polyspermic division takes place at the time of the first cleavage. However, if the eggs are re-fertilized after the full growth of the amphiaster, the process of the first cleavage goes on normally and polyspermic irregularity becomes visible at the second cleavage. It is of especial interest to note that the eggs are re-fertilizable even at the 2-cell stage if they have been allowed to develop in sea water after the treatment mentioned above. As shown in Figure 2, the eggs re-fertilized at the 2-cell stage become abnormal later on.

Isotonic solutions also of glycerine or thiourea are found to be effective for making the fertilized eggs re-fertilizable, while sea water containing ether, Cu-ion, trypsin, lipase, NaOH or HCl is not. Ca-Mg-free sea water becomes ineffective when any of Ca^{++}, Mg^{++}, Sr^{++} or Ba^{++} is added. It may be concluded that those media which contain any divalent cation are ineffective.

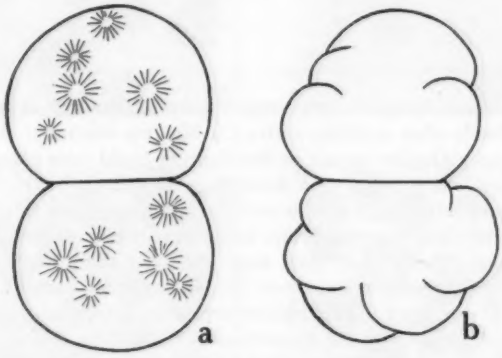

FIGURE 2. Eggs re-fertilized 5 minutes after the first cleavage. a. Thirty minutes after re-fertilization. Note many asters in each blastomere. b. One hour after re-fertilization. The second cleavage is polyspermic in each blastomere.

B. Experiments on Pseudocentrotus depressus, Heliocidaris crassispina and Temnopleurus toreumaticus

A further investigation of re-fertilization was made with three other species of sea urchins, its possibility being demonstrated to be fairly general. The three species used were *Pseudocentrotus depressus, Heliocidaris crassispina* and *Temnopleurus toreumaticus*. The procedure of the experiment was the same as was carried out with *S. pulcherrimus*. The fertilized eggs were put into Ca-Mg-free sea water and the removal of the fertilization membranes was performed in that solution. The results of the experiments, as summarized in Table V, show that all of the three species are capable of re-fertilization.

In several other experiments, Ca-Mg-free sea water was replaced with a molar urea solution with successful results.

Re-fertilization of the eggs without removal of the fertilization membrane

In recent years it has come to be recognized as a result of the works of Motomura (1941) and Runnström (1947) that the fertilization membrane is formed

TABLE V

Re-fertilization in several species of sea urchins. The fertilization membranes were removed in Ca-Mg-free sea water after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3})

Species	Temp.	Eggs 2-3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
P. depressus	18° C.	0%	39%	61%	0%
H. crassispina	26° C.	2	11	87	0
T. toreumaticus	26° C.	0	25	75	1

from the membrane which covers the unfertilized eggs and the material which exists below the protoplasmic surface in the unfertilized egg. On the basis of this fact it is obvious that the vitelline membrane is at first permeable to the spermatozoon but becomes impermeable as it turns into the fertilization membrane, though the so-called "wave of negativity" may have taken place earlier. For this reason, in the preceding experiments the fertilization membrane was mechanically removed before re-fertilization. The question arises as to the possibility of re-fertili-

TABLE VI

Re-fertilization of once-fertilized eggs without removal of the fertilization membranes

Medium in which eggs were immersed	Temperature	Time from insemination to immersion in Ca-Mg-free medium (seconds)	Duration of immersion in Ca-Mg-free medium (minutes)	Eggs 2 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
				Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10° C.	30	3	0%	6%	94%	1%
		50		0	15	85	
		90		0	99	1	
Urea solution (1 *M*)	11° C.	30	2	1	2	97	0
		50		1	4	95	
		80		0	4	96	
		120		0	93	7	

zation without removing the fertilization membrane when the hardening of the membrane has been inhibited. As stated before, it has been shown by several investigators that hardening of the fertilization membrane is dependent upon the presence of divalent ions such as Ca^{++} and Mg^{++} in the sea water, and lack of these ions causes a failure of hardening and thickening of the membrane (Hobson, 1932; Sugiyama, 1938a, 1938b). Accordingly an attempt was made to re-fertilize the once-fertilized eggs of *S. pulcherrimus* by the following procedure. The eggs are fertilized in the ordinary way and are put into a Ca-Mg-free medium just before the membranes begin to rise. In several minutes the membranes are elevated very high. When a molar urea solution is used the membranes become especially thin and delicate. Two to five minutes after, the eggs are put back into normal sea wa-

ter and sperm is gently added. The membranes sink to the surface of the eggs after being returned to sea water but they are not broken during this procedure. The count of polyspermic eggs is made after the first cleavage. Some of the results are shown in Table VI.

These results suffice to show that re-fertilization occurs without any mechanical removal of the membranes if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination. Too long a stay of the eggs in sea water before their removal into the Ca-Mg-free medium causes the failure of re-fertilization since the membranes become hard enough to prevent the penetration of the spermatozoa.

Discussion

It has long been accepted that fertilized eggs deprived of their fertilization membranes in normal sea water cannot be re-fertilized. This fact has been, as stated above, confirmed in the present work also. However, it has been found that fertilized eggs deprived of their membranes can, when washed in a Ca-Mg-free medium, be fertilized once again. It has also been demonstrated that when the hardening of the membrane has been inhibited, re-fertilization can take place without the removal of the membrane. In view of these findings it is justifiable to consider that in fertilized eggs there are two mechanisms of sperm exclusion. One of them is, of course, the fertilization membrane and the other is a certain substance covering the surface of the egg. The latter is so stable in sea water that it cannot be removed by any mechanical agitation. This is the reason why the removal of the fertilization membrane alone does not render the eggs re-fertilizable. However, it is noticed that this sperm-excluding mechanism becomes ineffective by washing with Ca-Mg-free sea water or non-electrolyte solutions. On the other hand, several kinds of reagents and solutions containing divalent cations were tried, but without success. These results seem to indicate that the sperm-excluding substance is soluble only in a medium which does not contain any divalent cation.

According to Just, the mechanism of monospermy in fertilization is attributed to the so-called "wave of negativity." The relationship between the wave of negativity and the sperm-excluding substance on the surface of fertilized eggs is an interesting problem. At present, however, no evidence has been furnished as to whether the sperm-excluding substance on the surface of the egg is the product of the wave of negativity or it is produced afterwards, independent of the wave of negativity.

In Urechis eggs, Tyler and Schultz (1932) have shown that the reversal of fertilization is possible by treatment with acid sea water. But with the eggs of the sea urchin no successful result has been achieved so far by means of acid sea water.

Bury (1913) reported that the fertilized eggs of the sea urchin could be fertilized again if they were exposed to a low temperature. A possible explanation of Bury's result is that the sperm-excluding substance on the surface of fertilized eggs may become ineffective at a low temperature, but a definite conclusion with respect to this point must await further investigation.

There have been several studies on the superposition of fertilization on parthenogenesis. Loeb (1913) found that the eggs of *Strongylocentrotus purpuratus,* in which membrane formation had been induced by butyric acid, could be fertilized by sperm if the membranes were torn by shaking. It was also found that the eggs

of the same form were fertilizable after activation by the treatment with hypertonic sea water. C. R. Moore (1916, 1917) showed that in the case of Arbacia there was no possibility of superimposing fertilization upon parthenogenesis after optimum exposure to the activating agent. Some other workers also came to a similar conclusion, using several kinds of marine eggs. Ishida and Nakano (1947, 1950) report that if the artificially activated eggs of *Strongylocentrotus pulcherrimus* are heavily inseminated after removal of the membranes, some of them can be fertilized, and that fertilization of the activated eggs is the more easily accomplished when the removal of the membrane is performed in Ca-Mg-free solutions. The results reported here on re-fertilization correlate closely with the findings of these two authors.

Attention may be called to the fact that re-fertilization can take place at any stage of the first cleavage or even at the 2-cell stage if the eggs have been allowed to develop in sea water after the removal of the fertilization membranes and their washing in a Ca-Mg-free medium. It would be of great interest to study the possibility of re-fertilization in the later developmental stages. Unfortunately, no experimental studies have yet been made on this problem.

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Summary

1. If the fertilized eggs of sea urchins (*Strongylocentrotus pulcherrimus, Pseudocentrotus depressus, Heliocidaris crassispina, Temnopleurus toreumaticus*) are inseminated again after removal of the fertilization membrane and washing with a Ca-Mg-free medium for a few minutes, re-fertilization can take place. Spermatozoa not only penetrate into the previously fertilized eggs but also take part in the formation of the mitotic figure, so that the cleavage of re-fertilized eggs is irregular and characteristic of polyspermy.

2. Re-fertilization can occur without removing the membrane mechanically if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination.

3. Re-fertilization can take place at any stage of the first cell division and even at the 2-cell stage.

4. It is justifiable to consider that there is a sperm-excluding substance on the surface of fertilized eggs. This substance is stable in sea water but is easily lost in Ca-Mg-free media.

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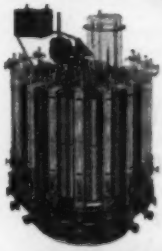

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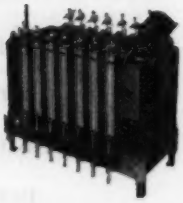

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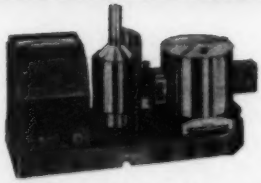

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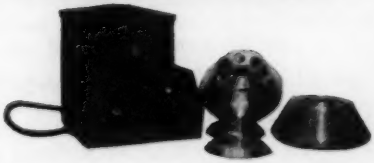

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